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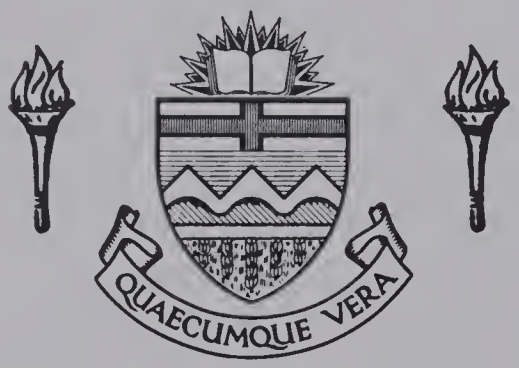

THE UNIVERSITY OF ALBERTA

STUDIES ON ETHYLENE BIOSYNTHESIS FROM ACRYLATE, β-ALANINE AND L-METHIONINE

by

© POONSOOK GHOOPRASERT

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE

DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF PLANT SCIENCE

EDMONTON, ALBERTA

FALL, 1971

Thesis
1976
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THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled "Studies on Ethylene Biosynthesis from Acrylate, β-alanine and L-methionine" submitted by Poonsook Ghooprasert in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

ABSTRACT

Elucidation of a biosynthetic pathway for the physiologically active gas, ethylene, has been the object of considerable experimentation. Many studies have been performed with intact tissues, tissue slices and subcellular particulate fractions. The purpose of this dissertation research was to contribute to existing information on possible precursors and pathways for ethylene biosynthesis.

A technique was developed for the preparation of a partially purified, soluble enzyme system from a subcellular particulate fraction of waxbean cotyledons (Phaseolus vulgaris). A 32,000 g fraction was rendered soluble by a 1 hr. treatment with 0.4% Triton X-100 followed with an 8 hr. treatment of the residual fraction with 0.1% Triton X-100. The combined soluble protein fraction was subjected to gel filtration on Sephadex G-50. The protein fraction that emerged from the column was active in synthesizing ethylene from acrylate in the presence of adenosine triphosphate, coenzyme A, a sulfhydryl protecting compound (dithiothreitol) and the cofactors for decarboxylation, thiamine pyrophosphate and magnesium ion. Further purification of the preparation by gel chromatography on Sephadex G-100 yielded preparations that had a sixfold increase in ethylene synthesizing activity per mg protein. The purified preparations could also catalyze ethylene production from pyruvate, propionate and β-alanine.

Although these purified enzyme preparations did not catalyze the conversion of L-methionine to ethylene, when bean leaves were fed with [U-^{14}C]-L-methionine, a substantial amount of [^{14}C]-ethylene was obtained. With [$^{14}CH_3$]-L-methionine, much less conversion to ethylene occurred; ^{14}C was found in great amount in carbon dioxide. Studies on metabolites extracted from all the leaf samples showed the presence of ^{14}C in orotic acid and uracil. These findings thus provide evidence for a conversion of L-methionine to orotic acid, uracil and ethylene.

Previous studies had shown that uracil could be converted to β-alanine and then to ethylene. These findings, along with the results of the present research indicate the existence of a pathway for the conversion of L-methionine to β-alanine. Both compounds have been shown by a variety of workers to be ethylene precursors. A solution to some of the controversy may thus be found in the demonstration of this pathway.

ACKNOWLEDGEMENTS

The work described in this thesis was carried out under the supervision of Dr. Mary Spencer. For her valuable guidance and unfailing encouragement throughout all phases of the research and manuscript preparation, I wish to express my sincere thanks and deepest gratitudes.

I would like to thank Dr. C. T. Phan for his helpful suggestions and interest in the work, Dr. Bakri and Mrs. Valerie Skura of the Food Science Department for their help with the gel electrophoresis, and all my colleagues in the South Lab for their moral support.

A special noteof thanks is extended to Mr. C. Benger and Mrs. Nora Everaars for their excellent technical assistance, and to Mrs. L. Pearl Williams for typing the thesis.

Financial support for one year from the National Research Council of Canada awarded indirectly through a research grant to Dr. Mary Spencer, and the two intersession bursaries from the University of Alberta are gratefully acknowledged.

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LIST OF ABBREVIATIONS

α-KG	α-ketoglutarate
ATP	adenosine triphosphate
BSA	bovine serum albumin
CoA	coenzyme A
cpm	counts per minute
dpm	disintegration per minute
DTT	dithiothreitol
GC	gas chromatograph
μc_i	microcurie
NADH	reduced nicotinamide adenine dinucleotide
Pal (P)	pyridoxal phosphate
RNA	ribonucleic acid
TCA	trichloroacetic acid
TES	N-tris(hydroxymethyl) methyl-2-aminoethanesulfonic acid
TLC	thin-layer chromatography
TPP	thiamine pyrophosphate
[U]	uniformly labeled

GENERAL INTRODUCTION

The elucidation of the biosynthetic pathway of ethylene from β-alanine was initiated in this laboratory. Evidence has been accumulated for the operation of the pathway and cofactor requirements, and the first enzyme on the pathway, β-alanine aminotransferase, has been characterized. The present research project centered on the purification and characterization of the last enzyme in the pathway, the enzyme catalyzes the conversion of acrylic acid to ethylene. In addition, methionine was tested as a precursor of ethylene, and certain relationships of methionine with β-alanine were explored.

REVIEW OF LITERATURE

A. Introduction

Ethylene production has been known to be rather general among living things (1). In higher plants various organs that have been found to produce the gas are leaves, both on the plant and off, (2, 3, 4), cotyledons (5), flowers (6), roots and tubers (7), germinating seeds and seedlings (8, 9, 10) and fruits (11, 12). The pathways of ethylene biosynthesis, however, are still not clear. Among the proposed precursors are β-alanine (13, 14), methionine (15, 16), linolenic acid (17), glucose (18, 19, 20), various acids of the tricarboxylic acid cycle (21) and ethanol (22). Details of reaction steps involved in each particular pathway have been thoroughly reviewed (1).

The biosynthesis of ethylene seems to depend a great deal on types of tissues, stages of their development and conditions of handling them. As an example, [^{14}C-2] β-alanine was found by Knight (2) to be converted to [C^{14}]-ethylene by detached leaves of waxbeans, but was found by Burg and Clagget (16) to be ineffective in apple tissue discs and green banana slices. The latter workers, however, made no mention of the concentration of the β-alanine used; also, green fruit does not normally produce appreciable ethylene.

Another example is the ethylene synthesis from methionine. Lieberman, Kunishi, Mapson and Wardale (15) found the amino acid to stimulate ethylene production from apple discs by 33 to 100%,

depending on the pH and buffer components and that both the D- and L-methionine were equally effective. The stimulation by methionine was observed only with postclimacteric tissue that had been stored for one month. Tissue slices from immature fruit, young fruit and freshly harvested mature fruit were not stimulated by methionine addition. Burg and Clagget (16) obtained [C^{14}]-ethylene from subapical sections of pea stem treated with 10^{-3} M indoleacetic acid prior to [U-C^{14}]-DL-methionine. In contrast to these findings, Galliard, Rhodes, Wooltorton and Hulme (23) found no stimulation of ethylene production by addition of methionine to either fresh or aged disks of the peel from preclimacteric apples. Lieberman et al (15) found the amino acid to be ineffective with tissue slices of tomato and avocado.

Complexities in elucidation of the pathway for ethylene biosynthesis are partly a result of the minute production of the gas by living organisms. In many cases, ethylene must be collected and concentrated before it can be detected, even by a very sensitive instrument now available (a gas chromatograph equipped with flame ionization detector that detects less than 10^{-11} moles of the gas (24, 25)). For concentration of ethylene a relatively long collection period would be necessary. Any observations made during this period, therefore, may not reflect the normal metabolic events of the tissues. This is especially true where radioactive precursors are used in a high concentration (a high activity of the compound administered could alter the physiological states and perhaps the biochemical processes of the tissues (26)).

Another problem that is encountered is the permeability barrier between certain compounds and their sites of metabolism in the tissues. Unless this information is available the comparison of the effectiveness of various compounds as 'active substrates' may not be meaningful.

To overcome the problem of permeability barrier and perhaps, but not necessarily, the question of the long reaction period, many workers have attempted to study the pathway of ethylene biosynthesis at subcellular and at enzyme levels. One criterion that may be used for assessment of an isolated enzyme system is that the amount of ethylene produced by the system should be high enough to account for the *in vivo* production of the gas from that tissue. However, there is ample evidence for the existence of more than one ethylene producing system, and they may even be operative to greater or lesser extents in the same tissue. It also should be borne in mind that an isolated enzyme or enzyme system may not behave the same way as it does in intact tissues in which many other factors controlling the metabolic patterns of that tissue play an essential role.

Chandra and Spencer isolated various subcellular fractions from tomato fruit in the advanced turning stage (27) and later from rat liver, rat intestinal mucosa and from *Penicillium digitatum* (28). They detected ethylene from the fraction separated by centrifugation at 35,000 *g* for 15 minutes after the preparation had been sonically treated or aged *in vitro*. Mapson and Wardale (29) isolated an

enzyme system from cauliflower florets that catalyzed production of ethylene from methionine. Abeles and Rubinstein (30) prepared from etiolated pea seedlings a cell-free extract that catalyzed ethylene production from an endogenous, extractable substrate from the same tissue.

It should be pointed out here that many nonenzymic model systems have been proposed and investigated for the production of ethylene from various precursors (see eg. 17, 31, 32, 33). Their significance *in vivo* is difficult to assess but insofar as an isolated enzyme system is concerned conditions for assaying the ethylene synthesizing activity should be so selected that contribution from those nonenzymic model systems is kept at the minimum.

B. Ethylene Synthesis by in vitro systems

1) Pyruvate as precursor

Shimokawa and Kasai (34) found radioactivity in ethylene after an unstated period of incubation of a subcellular fraction (obtained by centrifugation at 13,000 *g* for 20 minutes) of apple tissue with [^{14}C-3]-pyruvate and cofactors (magnesium ion and thiamine pyrophosphate). They identified a labeled intermediate in the reaction flask, after an incubation period of 30 minutes, as acetaldehyde. Since their earlier work showed a direct conversion of acetaldehyde-cysteine complex (quoted by Shimokawa and Kasai (32)) into ethylene (the reaction that was nonenzymic and was mediated by light and flavin mononucleotide) they proposed the conversion of pyruvate to ethylene as being the result of two

reaction steps, an enzymic decarboxylation of pyruvate to acetaldehyde; and a nonenzymic conversion of acetaldehyde to ethylene. No mention was made in their paper, however, about the assay condition requiring light nor the presence of cysteine in the assay mixture for the enzyme preparations.

2) Linolenic acid as precursor

Galliard, Rhodes, Wooltorton and Hulme (35) prepared a tissue homogenate from apple peels, which, when incubated with linolenic acid and ascorbic acid produced both ethylene and ethane. The ratio of the two gases produced was 1:2 in air but it was 2:1 in oxygen. A nonenzymic system has been described by Lieberman and Mapson (17). According to this model system ethylene and a small amount of ethane were produced during the cuprous ion catalyzed degradation of peroxidized linolenic acid or a variety of other compounds having either an epoxy or ethoxy group in their molecules or having the terminal CH_3-CH_2-CH= group. In the system of Galliard et al a lipoxidase enzyme appeared to be a necessary requisite. A rise in lipoxidase activity was found by Meigh, Jones and Hulme (36) to precede the evolution of ethylene and reached a maximum just before the natural ethylene evolution peak. They pointed out that this was consistent with a role for lipoxidase in ethylene biosynthesis, possibly in promoting the decomposition of linolenate.

3) Methionine as precursor

Mapson and Wardale (29) isolated an enzyme system from cauliflower florets, and later from half-ripe tomatoes (37). Both systems showed the following enzyme activities (37, 38):

a) A transaminase that was located in a particulate fraction, possibly a mitochondrial-microsome fraction. The enzyme catalyzed conversion of methionine to 4-methylmercapto-2-oxo-butyric acid. In this transamination reaction, pyridoxal phosphate was required as a cofactor and phenylpyruvic acid was an amino acceptor.

b) Glucose oxidase and a peroxidase that were localized in the nonparticulate fraction and catalyzed the breakdown of the oxobutyric acid compound or methional, another possible product of the reaction in (a), to ethylene. These workers thought the role of glucose oxidase system was to provide hydrogen peroxide, which was subsequently used for generation of ethylene.

c) A peroxidase system requiring a phenolic compound, β-hydroxybenzoate and an acidic compound, sulphinic acid that could be replaced by sulphite ion.

In the system of tomato tissues, the glucose oxidase system was replaced by a lipoxygenase-linolenic acid system. The localization and requirement for pyridoxal phosphate of the transaminase was different from that of cauliflower floret system (37).

In further investigation of the role of the glucose oxidase system in the production of ethylene from methionine, Lund and Mapson

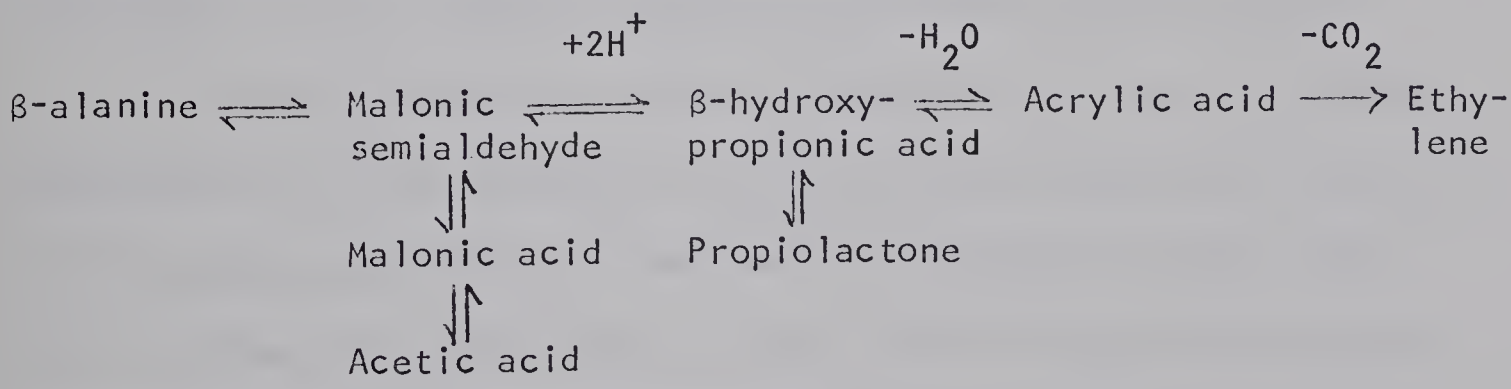
(39) found that there was a marked increase in ethylene production of the floret tissues when they were inoculated with the soft-rot bacterium Erwinia carotovora. They claimed that this increase in ethylene production was the result of increase in the glucose oxidase activity of the floret tissues, consequently, an increase in hydrogen peroxide, which was thought to be a limiting factor in the production of ethylene from methionine. However, there was no direct evidence presented in their paper for the increase in hydrogen peroxide.

Demorest and Stahmann (40) obtained ethylene when D,L-methionine was incubated in a system containing resorcinol, horseradish peroxidase, magnesium ion and bisulphite ion at pH 7.8. From the system in which methionine was replaced with peptides containing methionine, or methionine derivatives at the carboxyl end, the initial rate of ethylene production was two to four times as high as that obtained with free methionine. The peptides containing methionine internal residue or methionine at the amino end, however, produced little or no detectable ethylene, but the N-formyl D,L-methionine and the N-acetyl-D,L-methionine were equally and much more active, respectively, in producing ethylene than the peptides with a C-terminal methionine residue. From these results and their finding that egg albumin evolved ethylene from the system only after it had been treated with a proteolytic enzyme, pronase, the workers suggested the role of N-acylated derivatives of methionine as a precursor of ethylene in plant tissues, and the role of proteolytic

enzymes in increasing the ethylene synthesizing activity of plant tissues that had undergone mechanical injury or fungal infections.

4) β-alanine as precursor

The work of Chandra and Spencer (27, 28) on localization of the active site for ethylene synthesis in tomatoes, rat liver, rat intestinal mucosa, and Penicillium digitatum was extended by Thompson and Spencer (5) to cotyledons of the waxbeans Phaseolus vulgaris. Partially purified enzyme powders were prepared from the 25,000 g fraction. In the presence of appropriate cofactors, the enzyme powders converted [^{14}C-2]-β-alanine to radioactive ethylene. Subsequent work by the same workers (14) to identify and to quantitatively determine the labeled intermediates led them to propose the following pathway for conversion of β-alanine to ethylene:



A reversal of the equilibrium between malonic semialdehyde and malonate, in favour of the former, was believed to be responsible for the 83% stimulation of ethylene production from β-alanine when malonate was added. The role of malonate in 'forcing' the equilibrium towards ethylene production was later investigated and confirmed by Stinson (41). The proportion of ethylene derived from

β-alanine (obtained by dividing the specific activity of the ethylene by that of β-alanine) was found to be 0.57 indicating that the conversion route to ethylene was fairly direct (14). A possible explanation for the low percentage of conversion of administered [^{14}C-2]-β-alanine to [^{14}C]-ethylene became more obvious after the finding of Knight (2) that detached bean leaves at the age when maximal ethylene evolution was observed had a large pool of β-alanine.

According to the scheme proposed, the water medium would have an important role. A study of Stinson (42) revealed that protons from the medium containing tritiated water were incorporated into the ethylene molecule. It was possible that one proton on each carbon or if there was an equilibrium between each step and the proton source in the medium where tritinum was present, all the four 'hydrogens', of the ethylene molecule would have become labeled.

From the proposed scheme of Thompson and from the studies on effects of various cofactors on stimulation of ethylene production by the enzyme powders and by the tomato particulate fraction (13), it has become evident then that at least the following four enzymes are involved in the ethylene synthesis from β-alanine:

a) A transaminase that catalyzes the conversion of β-alanine to malonic semialdehyde.

b) A dehydrogenase that catalyzes the reduction of malonic semialdehyde to β-hydroxypropionic acid.

c) An enoyl hydrase that catalyzes the dehydration of β-hydroxypropionic acid to acrylic acid.

d) A decarboxylase that catalyzes the decarboxylation of acrylic acid to ethylene.

The β-alanine aminotransferase was identified by Stinson and Spencer (43) as a constituent of the preparation that was rendered soluble from the 32,000 g fraction from waxbean cotyledons. Either oxaloacetate or pyruvate could act as amino acceptor, but the aminotransferase activity was fifteen times more reactive with oxaloacetate. The enzyme showed a requirement for pyridoxal phosphate as shown by a 40% stimulation of activity on addition of 1×10^{-4} M of the cofactor.

The other two enzymes on the pathway, dehydrogenase and enoyl hydrase have been identified in animals (44, 45), micro-organisms (46, 47), and to a much less extent in higher plants (48, 49). In a preliminary investigation reported by Rendina and Coon (45) there appeared to be a specific NAD-dependent enzyme of pig kidney that catalyzed dehydrogenation of β-hydroxypropionate to furnish malonic semialdehyde. In the same report the workers found the equilibrium for hydration of acrylyl CoA to β-hydroxypropionyl CoA to favour acrylyl CoA and that hydration could be demonstrated only when β-hydroxyproprionyl CoA was removed. In their experiment the enzyme crotonase was used.

Giovanelli and Stumpf (48) in a study of fat matabolism found the oxidation of propionate to occur first by the formation of propionyl CoA in the presence of ATP, coenzyme A and α-keto-glutarate and oxygen. The second step of conversion was the

dehydrogenation of propionyl CoA to acrylyl CoA followed with hydration of acrylyl CoA to β-hydroxypropionyl CoA, which was further metabolized via unidentified intermediates to carbon dioxide. Calleley and Lloyd (50) and Lloyd and Venables (51) found in the alga Prototheca zopfii enzymes capable of linking the intermediates malonic semialdehyde, β-hydroxypropionate, and acrylate of the proposed biosynthetic scheme.

The conversion of acrylate to ethylene in Penicillium digitatum was reported by Jacobson and Wang (52). When the radioactive acrylate was administered to the mold, the distribution of ^{14}C was found to be as summarized in Table 1.

In the presence of an inhibitor cis-3-chloroacrylic acid at 10^{-3} M concentration, the workers found the ethylene production was inhibited almost completely.

Recently, Shimokawa and Kasai (53) reported the ethylene formation from acrylic acid by an extract of banana pulp. It was necessary, however, to conduct the reaction under an inert gas argon, which is not normally present in large amount inside any cells and tissues. The decarboxylation of acrylic acid was found to require the presence of thiamine pyrophosphate and magnesium ion.

The role of acrylate in ethylene biosynthesis was postulated earlier by Varner (54). Phan (55) found 50 mM acrylate to be toxic to petunia flowers. Meheriuk (13) observed the inhibition by acrylate at 50 mM on ethylene production from a tomato particulate fraction. Thompson (56)

Table 1

Distribution of ^{14}C in carbon dioxide and ethylene produced by Penicillium digitatum administered with [^{14}C]-acrylate (52)

Compound	Radioactivity	Amount of substrate	% Distribution radioactivity		
	(μc)	(μmole)	$^{14}C_2H_4$ ($\times 10^{-2}$)	$^{14}CO_2$	Media
Acrylate-1-^{14}C	3.68	5	0.1	82	12
Acrylate-2-^{14}C	2.50	5	16.1	27	22
Acrylate-3-^{14}C	2.50	5	2.7	36	21

found an inhibitory effect of acrylate on ethylene production by enzyme powders from wax bean cotyledons. At 25mM concentration of acrylate the inhibition was 25% and at 75 mM the inhibition was 70%.

Stinson (42) was able to show a slight stimulation of ethylene production by acrylate added to soluble enzyme system prepared from the 32,000 g fraction of wax bean cotyledons. The ethylene production from the system containing 0.2 mM acrylate was increased by 38% over the system containing no acrylate. Addition of dithiothreitol (DTT) at 9.7 mM to the assay mixture enabled the enzyme to catalyze ethylene synthesis from acrylate at 0.5 mM giving as much as three times the amount of ethylene as synthesized in the absence of DTT.

C. Ethylene Synthesis and Bean Leaves

Stinson (42) demonstrated that excised primary leaves of wax beans, grown in California soil mix in a 14 hour light day at 65°F and a light intensity of 1200 ft. c, at an age between 16-19 days evolved considerable amounts of ethylene. When [U-^{14}C]-L-methionine (17-15 μc/μmole) was administered to the 19 day old leaves, and the collection of ethylene was delayed until 4 hours after incorporation of the radioactive methionine, the specific activity of the evolved ethylene was almost four times greater than when collection was delayed only 1 hour. Thus, he concluded that the methionine incorporation might become more prominent with time.

Knight (2) in the study on RNA metabolism in relation to

ethylene production of bean leaves (grown under similar conditions to those of Stinson) found production of [^{14}C]-ethylene from [^{14}C-2]-β-alanine. He also showed that labeled β-alanine could in turn be produced by administration of [^{14}C-6]-orotic acid or [^{3}H-C_6]-uracil to detached bean leaves. Since bean leaves at those ages showed a decrease in the total RNA content, (which might result from a decreased RNA synthetic activity of the leaves as well as an increase in degradation rate of RNA) he proposed linkages between RNA metabolism and β-alanine and ethylene production, through the formation of uracil.

It may be noted here that bean leaves that were used by the two workers were harvested from the plants grown under the same conditions, and were at the same or similar stages of development (since they were at the same age). It is possible that some interesting interrelationships may be existing for the ethylene synthesis from L-methionine and from β-alanine in these leaf tissues.

In the present research project, improved conditions were sought for the preparation and assay of the enzyme preparations from wax bean cotyledons. Increased activity in the conversion of acrylate to ethylene was sought through application of various purification procedures. Investigations of the properties of purified enzyme preparation, insofar as practicable was planned.

In addition, tracer studies were made with wax bean leaves fed with [C^{14}]-L-methionine to investigate a possible relationship between metabolism of this amino acid and that of β-alanine for the ethylene production.

MATERIALS AND METHODS

A. Sources of Chemicals

Routine chemicals (all reagent grade) were from Fisher Scientific Co., Ltd. Biochemicals were generally of the highest purity available and obtained from the following sources: [U-^{14}C]L-methionine (>99%), [$^{14}CH_3$]-L-methionine (>99%), PPO (2,5-diphenyl-oxazole) and POPOP (p-bis-2-(5-phenyloxazoloyl)-benzene) from New England Nuclear; [U-^{14}C] ethylene (>98%) from Nuclear Chicago Corporation; ATP, β-alanine, α-KG, dithiothreitol, from Calbiochem.; TES, NADH, Coenzyme A, thiamine pyrophosphate, pyridoxal phosphate from Sigma Chemical Company; sodium acrylate from K and K Co., Sunset Blvd., Hollywood, California; Triton X-100 (octylphenoxypoly-ethoxyethanol) from Applied Science Laboratories; ethylene (USP 99%) from Ohio Chemical and Manufacturing Company; Sephadex G-50, G-100 and G-200 from Pharmacia.

B. Tissues for studies

Seeds of waxbean Phaseolus vulgaris var. Kinghorn were used. Seeds were soaked in tap water for four hours. Then seeds that were fully swollen and the ones in which imbibition of water had not begun were selected out. The remaining seeds were planted in vermiculite and germinated in the dark at 27°C for studies on cotyledons. One day old cotyledons were the cotyledons harvested twenty-four hours after planting.

Seeds that were used for studies on bean leaves were grown in vermiculite at 25°C, 50% humidity and 14 hour-day of about 1800 foot-candles. Only the first two leaves were used in all experiments.

C. Collection of Ethylene (after Knight (2))

A continuous stream of compressed air was freed of ethylene by passing through mercuric perchlorate adsorbed on silica gel (28-200 mesh) in a U-tube that was placed in an ice bath (this air will be called 'purified air' for simplicity). The 'purified air' flowed into reaction flasks containing the material under study, and out with other gases evolved from the reaction flask through the following series of U-tube (ID 1.5cm): a tube containing lithasorb to remove carbon dioxide; a tube containing drierite to remove moisture, and a cold trap tube as a coolant. The gas stream was then passed through 0.5 g silica gel (28-200 mesh), and ethylene was quantitatively adsorbed on the silica gel in a U-tube (ID 3.5 mm). The gas mixture evolved from whole cotyledons was swept out of the flask containing tissues at a flow rate of 50 ml per minute, and at 30 ml/min and 100 ml/min from enzyme preparations and from bean leaves respectively.

Immediately prior to collecting ethylene, the small U-tube containing 0.5 g silica gel was heated in a boiling water bath for approximately 20 minutes. During this heating period, a stream of 'purified air' was passed continuously through the tube to remove

all the gases that were evolved from silica gel. The tube was then sealed with a short piece of rubber tubing and transferred to the collection system. After the collection period the tube was disconnected from the cold-trap tube and again sealed with rubber tubing and kept in dry ice-acetone bath. Measurement of the ethylene was done immediately.

When bean leaves were used, they were cut under water, close to the main stem. Leaves were placed with petioles in a small beaker of water, which was then placed in a reaction flask, and ethylene collection was commenced immediately. Three to four leaves were found to evolve sufficient ethylene for analysis on the gas chromatograph.

When radioactive compound was used to feed into bean leaves, the solution of radioactive compound was put in a small vial (capacity of approximately 0.7 ml) and three leaves were placed with petioles in this solution. A small amount of drierite was placed underneath the vial to hasten uptake of radioactive solution (2). In these feeding experiments, the U-tube containing lithasorb was replaced with 2.0 ml of 3% (w/v)KOH(2).

The gas collection period for all the experiments was 0-4 hours (2, 42). All reaction flasks were covered with aluminum foil.

D. Ethylene Analysis (after Knight (2))

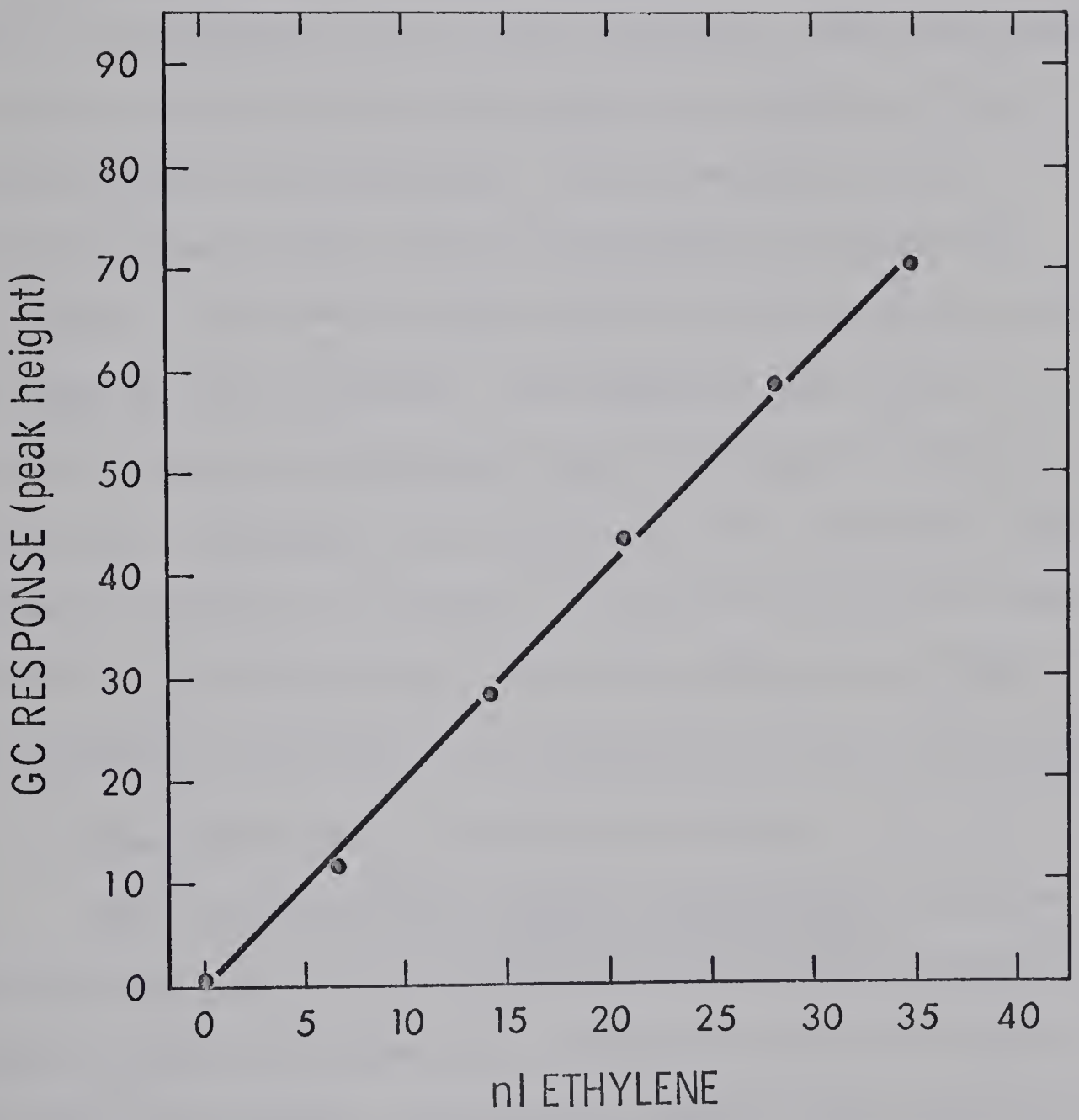
The silica gel tube disconnected from the cold trap at the end of a collection period was connected to a two-way valve

inserted into the helium line of a Perkin Elmer Model 811 flame ionization gas chromatograph. The valve was opened and the U-tube, which was still in dry ice-acetone bath, was flushed with helium for 50 seconds with one end open to atmosphere to remove all the gas residues from the tube and from the dead space of the injection port of the gas chromatograph. This was followed by a 10 second flushing with the other end connected to the helium input line into the GC. With the valve closed, the U-tube was heated in a water-bath at 43° for 3 minutes to release gases from the silica gel. The valve was then opened and the contents of the U-tube were flushed onto a 50 cm x 6 mm. (internal diameter) column of activated alumina coated with 2 1/2% silicone 550. The column was operated at 20° and the temperature of the detector was 125°. Under these operating conditions, four peaks were obtained from gas samples. The first and the fourth peaks have not yet been identified with certainty, but the second peak has been identified as ethane and the third peak as ethylene.

The heights of ethylene peak were calibrated against known amounts of standard ethylene in nl. With this calibration curve (fig. 1, p. 20) the amount of ethylene in an unknown sample could be determined from the peak height of that sample.

For an estimation of [^{14}C]-ethylene, the gas chromatograph was connected to a Nuclear-Chicago gas flow counter. In so doing approximately 4/5 of the total gas mixture was diverted from the gas chromatograph.

Figure 1: Ethylene standard curve.
The volatile was subjected to gas chromatography and was resolved by a column (50 cm x 6 mm ID) of activated alumina with 2 1/2% silicone 550.
He, H_2, and air flow rates were 64 cc/min, 37 cc/min, and 200 cc/min, respectively.
Column temperature was approximately 20° and detector temperature was approximately 125°.


GC RESPONSE (peak height)
0
10
20
30
40
50
60
70
80
90
0
5
10
15
20
25
30
35
40
nl ETHYLENE

E. Preparation of the Ethylene synthesizing Enzyme system from Bean Cotyledons

1) The lyophilized 32,000 g preparation (after Stinson (42))

Bean cotyledons were washed with deionized water and chilled in a refrigerator for at least 30 minutes. They were ground in a chilled meat-grinder while the medium for isolation of sub-cellular particles was being added. The medium was 0.3 M in mannitol and 50 mM in TES, with the pH adjusted to 7.4 (at 0°C) with 1N NaOH. Volume of the medium added was twice as much as the fresh weight of bean cotyledons. The homogenate was filtered through four layers of cheesecloth, then centrifuged at 2,500 g for 10 minutes. Subsequent centrifugation of the supernatant layer was done at 32,000 g for 15 minutes. The particulate fraction was suspended in 10 mM TES adjusted to pH 7.6 (at 0°C) with 1N NaOH (1.0 ml buff/8 g. cotyledons). The suspension was then lyophilized.

2) Enzyme preparation by freezing and thawing

20 ml. of the 32,000 g fraction (prepared as in (1)) in before lyophilization, 10 mM TES buffer, pH 7.6 (at 0°)/was quickly frozen by immersing the tube in a dry-ice acetone bath. Thawing was done in a water-bath at 40° for 5 minutes. Freezing and thawing of the enzyme preparation was done three times before assaying for the ethylene synthesizing activity.

3) Soluble enzyme preparation

The method of Stinson (41) was used with slight modifications. The lyophilized enzyme preparation from (1) was suspended and stirred

gently for one hour in 0.4% Triton X-100 prepared with 10 mM TES, pH 7.6 (at 0°) (1 g powder/17 ml Triton solution). The suspension was then centrifuged at 100,000 g for 30 minutes. The top whitish layer (possibly of lipids) was removed and the supernatant layer was desalted by passing through a column of Sephadex G-50 (K 25/45) at the flow rate of 60 ml/hr. The eluting buffer was 10 mM TES, pH 7.6 (at 0°) containing 0.1% Triton X-100. The sediment from centrifugation at 100,000 g for 30 minutes was suspended in 0.1% Triton X-100 in 10 mM TES, pH 7.6 at 0° (same volume as the 0.4% Triton solution) and stirred gently for 8 hours. Subsequent centrifugation and desalting were done as before. The fractions of soluble protein that were eluted from the Sephadex column were combined and lyophilized.

The whole operation was done at temperature between 0° - 4°.

4) Ammonium sulphate fractionation of soluble protein preparation

Fractions of soluble protein from (3) were combined and brought to the desired percentage saturation of ammonium sulphate by addition of solid ammonium sulphate (57) at 0° - 4°. Fifteen minutes after addition of ammonium sulphate the solution was centrifuged at 10,000 g for 10 minutes at 2° to sediment the protein precipitates.

5) Sephadex gel filtration of the soluble protein preparation

0.3 g of lyophilized soluble protein from (3) was dissolved in 3.0 ml. 10 mM TES pH 7.6 (at 0°). Elution of the fractions was

performed in a downward manner at flow rate of 12 ml/hr from Sephadex G-200 column and at flow rate of 20 ml./hr from Sephadex G-100 column. Fractions of 2.2 ml. were collected. Protein fractions were monitored with an LKB Model 4701 A (Uvicord 1) at wavelength 254 nm.

F. Gel Electrophoresis of the Purified Preparations

The protein fraction that was active in synthesizing ethylene from acrylate was analyzed for purity by gel electrophoresis in an alkaline and an acidic medium.

1) Disc gel electrophoresis in an alkaline medium

The method was slightly modified (Bakri - personal communication) from the method of Davis (58). Stock solutions are:

Buffer A:	1 NHCl	48.0 ml
	TRIS	36.6 g
	TEMED	0.23 ml. (TEMED = N,N,N^1,N^1-tetramethylethylenediamine)
	Water to 100.0 ml. pH 8.9 at 25°	
Buffer B:	1 NHCl	48.0 ml.
	TRIS	5.98 g.
	TEMED	0.46 ml.
	Water to 100.0 ml. pH 6.7 at 25°	
Solution C:	Acrylamide	28.0 g.
	BIS	0.735 g
	Water to 100.0 ml.	

Solution D: Acrylamide 10.0 g.

BIS 2.5 g.

Water to 100.0 ml.

Solution E: Riboflavin 4 mg.

Water to 100.0 ml.

Solution G: Ammonium persulphate 0.6 g.

Water to 100.0 ml.

Electrode buffer (H): TRIS = 6.0 g.

Glycine = 28.8 g.

H_2O to 1 litre pH 8.3 at 25°

Stock solution was diluted 1:1 for the upper reservoirs, and 1:10 for the lower reservoir.

The following ratios were used for:

a) separating gel

1 part A : 2 parts C : 1 part water (or 8 M urea). This solution was combined with catalyst G in 1:1 ratio.

b) stacking gel

1 part B : 2 parts D : 1 part E

c) sample gel

4 parts protein sample : 8 parts D : 1 part B : 3 parts E

Electrophoresis was carried out at room temperature for 2 1/2 hours with a constant current of 5 mA per tube. The lower reservoir served as the anode.

2) Gel electrophoresis in an acidic medium (59)

The gels contained 7.5% (w/v) acrylamide 35% (v/v) acetic acid, and 5 M urea. For the preparation of the gels, 3 ml. of a stock solution containing 0.3 g of acrylamide, 0.6 g. of urea, and 0.008 g. BIS in 47% (v/v) acetic acid were mixed with 1 ml. of a fresh solution containing 0.6 g. of urea, 0.015 g of ammonium persulphate, and 0.02 ml. of Temed. The protein solution (100 µl containing 200 to 400 µg of protein) was mixed with 50 µl of a 40% (w/v) sucrose solution in 35% (v/v) acetic acid and was put on top of the gel. Both upper and lower reservoirs of the electrophoresis apparatus were filled with 10% (v/v) acetic acid. The lower reservoir served as a cathode, and electrophoresis was carried out at room temperature for 3 hours at a constant current of 5 mA. per tube.

Both alkaline and acidic gels were stained with 1% Amido black in 7% (v/v) acetic acid for 30 minutes in the former case and 1 hour in the latter. Destaining of excess dye was done by immersing the gels in 7% (v/v) acetic acid for at least 24 hours.

G Assay Mixtures for the Enzyme Preparations

1) Ethylene synthesis from β-alanine

A complete assay medium (41) was:

50 mM in β-alanine; 50 mM in malonic acid; 50 mM in α-ketoglutaric acid; 1.5 mM in ATP; 2.0 mM TPP; 1.0 mM $MgSO_4$; 0.5 mM pyridoxal phosphate and 0.40 in mM NADH. The solution was made up in 10 mM TES adjusted pH to 7.2 with 1 N NaOH.

2) Ethylene synthesis from acrylate

A complete assay medium was 1.0 mM in sodium acrylate; 1.5 mM in ATP; 2.0 mM in TPP; 1.0 mM in $MgSO_4$; 0.17 mM in CoA and 6.5 mM in dithiothreitol (DTT). The solution was made up in 50 mM TES adjusted pH to 7.2 with 1 NNaOH.

Along with those complete assay systems, ethylene was measured from the system containing no added substrate (blank system). The ethylene from complete system, after subtraction of ethylene from the blank system, was taken as the measure of enzyme activity towards the added substrate.

All the assays were done at 27° - 28° for the period of 0 - 4 hours.

H. Quantitative Determination of Protein

After the enzyme activity had been assayed the protein content of each flask was precipitated with 10% trichloroacetic acid. The protein precipitates, after centrifugation at 1200 g for 15 minutes, were dissolved in a minimum amount of 1 NNaOH, and the

estimation of the protein was done by the Lowry method (60) with the following modifications:

1) The solution of sodium carbonate was made up with water instead of with 1 NNaOH.

2) The final amount of NaOH in each protein solution was kept to 500 µmole to obtain the maximal intensity of the colour of the final mixture (61).

The other reagents and the time for colour development were the same as those described by Lowry et al. Absorbance was measured at 750 nm in a Beckman Model B spectrophotometer.

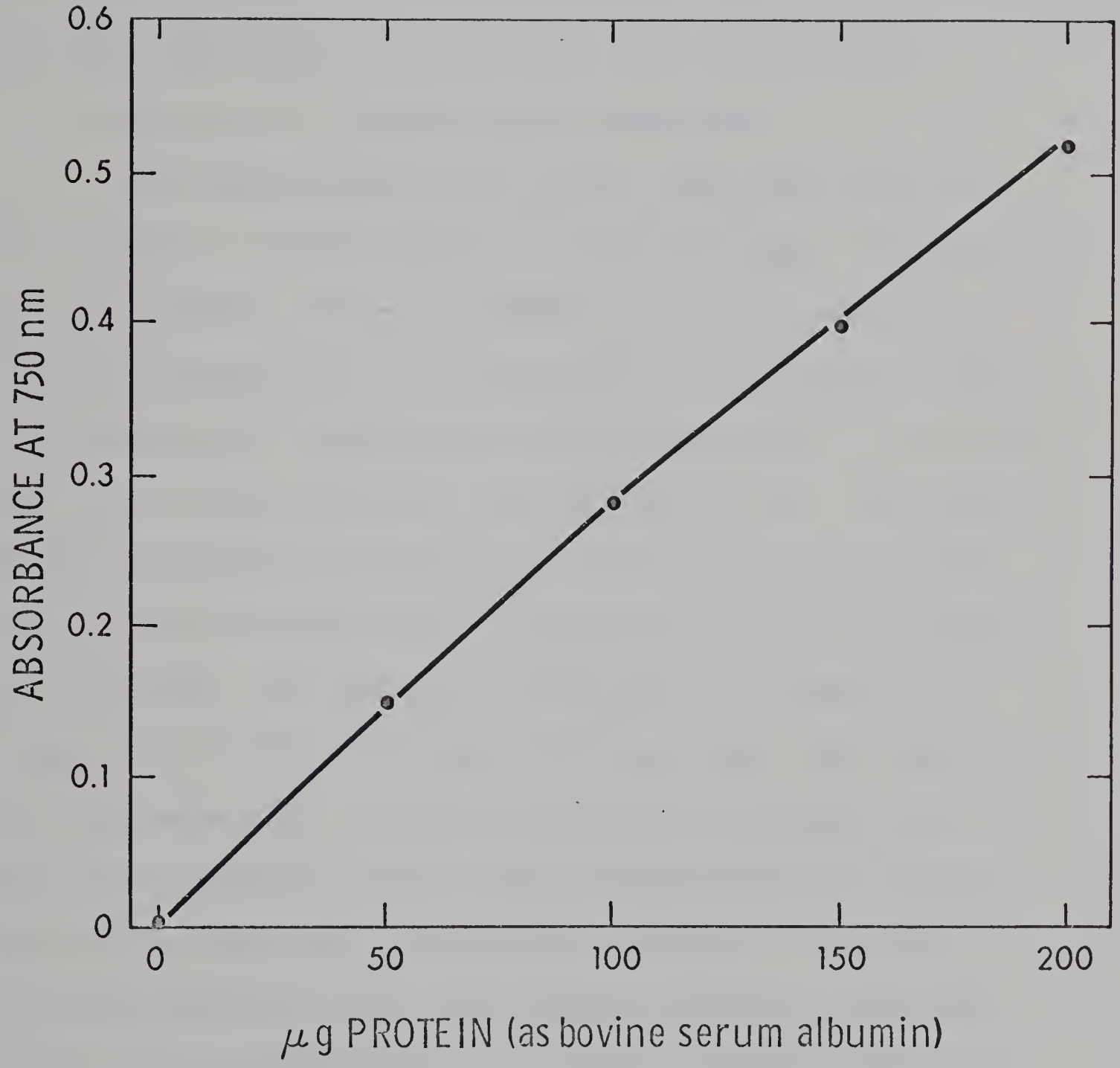
Bovine serum albumin in water was used as a standard and treated similarly. (For the protein standard curve see fig. 2, p. 28).

I. Extraction and Identification of Metabolites from Bean Leaves

1) Extraction of metabolites

The method described by Knight (2) was used. Leaves stored in dry-ice for at least two hours were powdered with pestle in a mortar. After the powder had been warmed up to room temperature (time taken was approximately 15 minutes) the grinding and subsequent extractions were made first with boiling 80% ethanol (35 ml/g. fresh weight) for 3 minutes, then twice with the same volume of boiling water for 5 and 3 minutes respectively. After each extraction the mixture was centrifuged at 3000 g for 5 minutes at 4°. The

Figure 2. Protein standard curve (Lowry et al, 1951) Amount of protein expressed as total protein in the solution mixture.


0.6
0.5
0.4
0.3
0.2
0.1
0
ABSORBANCE AT 750 nm
0
50
100
150
200
μg PROTEIN (as bovine serum albumin)

supernatant layers were combined and evaporated to dryness. Three ml. of water was added to the residue. After thorough mixing it was centrifuged at 26,000 g for 10 minutes at 4°. The clear supernatant layer was used for spotting TLC plates. (In each extraction there was a small amount of chlorophyll left in the residue).

2) Extraction of ribonucleic acid (Knight (2))

The residue after extraction with 80% ethanol (seel 1) was suspended in 1 M NaCl (10 ml./ g. original tissue). The suspension was heated rapidly to 90° for 7 minutes then rapidly cooled to 0° in a dry ice-acetone bath. The suspension was centrifuged at 3000 g for 15 minutes at 0° and potassium hydroxide was added to the supernatant layer to give the final concentration of 1.0 M. The solution was left overnight at room temperature then cooled in an ice-bath. To this solution 0.25 ml. of 0.05 M magnesium chloride was added per 12 ml. solution. Centrifugation at 3000 g for 15 minutes at 0° was done after addition of 2.0 ml. 11.8 M perchloric acid. The clear supernatant layer containing hydrolysed nucleosides was measured for radioactivity in a Nuclear Chicago Unilux II liquid scintillation counter (2). Scintillation fluor was: 7.0 g PPO (2.5-diphenyloxazole), 0.3 g. POPOP (p-bis-2-(5-phenyl-oxazolyl)-benzene), 100 g naphthalene and 1 litre of 1,4-dioxane. Ten ml of fluor was added per vial.

3) Identification of radioactive metabolites by TLC

The method described by Knight (2) was used.

One hundred µl of metabolites extract from I (1) was spotted as a band on a TLC plate coated with MN cellulose powder 300. The plate was developed with a solvent system consisting of isopropanol-pyridine-water-acetic acid (8:8:4:1 v/v/v/v) (62). Radioactive spots were located by scanning the plate through an actigraph (Model III - Nuclear - Chicago equipped with Thin-Layer-Plate Conveyor Systems Model 1006) set with the collimator slit width of 1.5 mm., time constant 5 seconds, scan speed of 30 cm/hr (for optimal operation). The operating voltage was 980 volts. Quench gas was a mixture of 98.7% helium and 1.3% butane regulated to give the pressure of 10 p.s.i.

The radioactive spots were scraped off the cellulose plate and extracted with 10.0 ml. cold water followed by extraction with 10.0 ml. boiling water. The two extracts were combined and evaporated to dryness. Hot deionized water (0.3 ml.) was added to the residue and the solution was used for rechromatography on the TLC. For this 100 µl. was spotted as a band on a plate coated with cellulose or silica gel G. The cellulose plate was developed with a solvent system for nitrogenous bases (63), which was n-butanol-methanol-water-ammonia (60:20:20:1 v/v/v/v). The silica gel plate was developed with a solvent system for amino acids (64), which was 96% ethanol-34% ammonia (70:30 v/v). Radioactive spots were detected with the actigraph.

RESULTS AND DISCUSSION

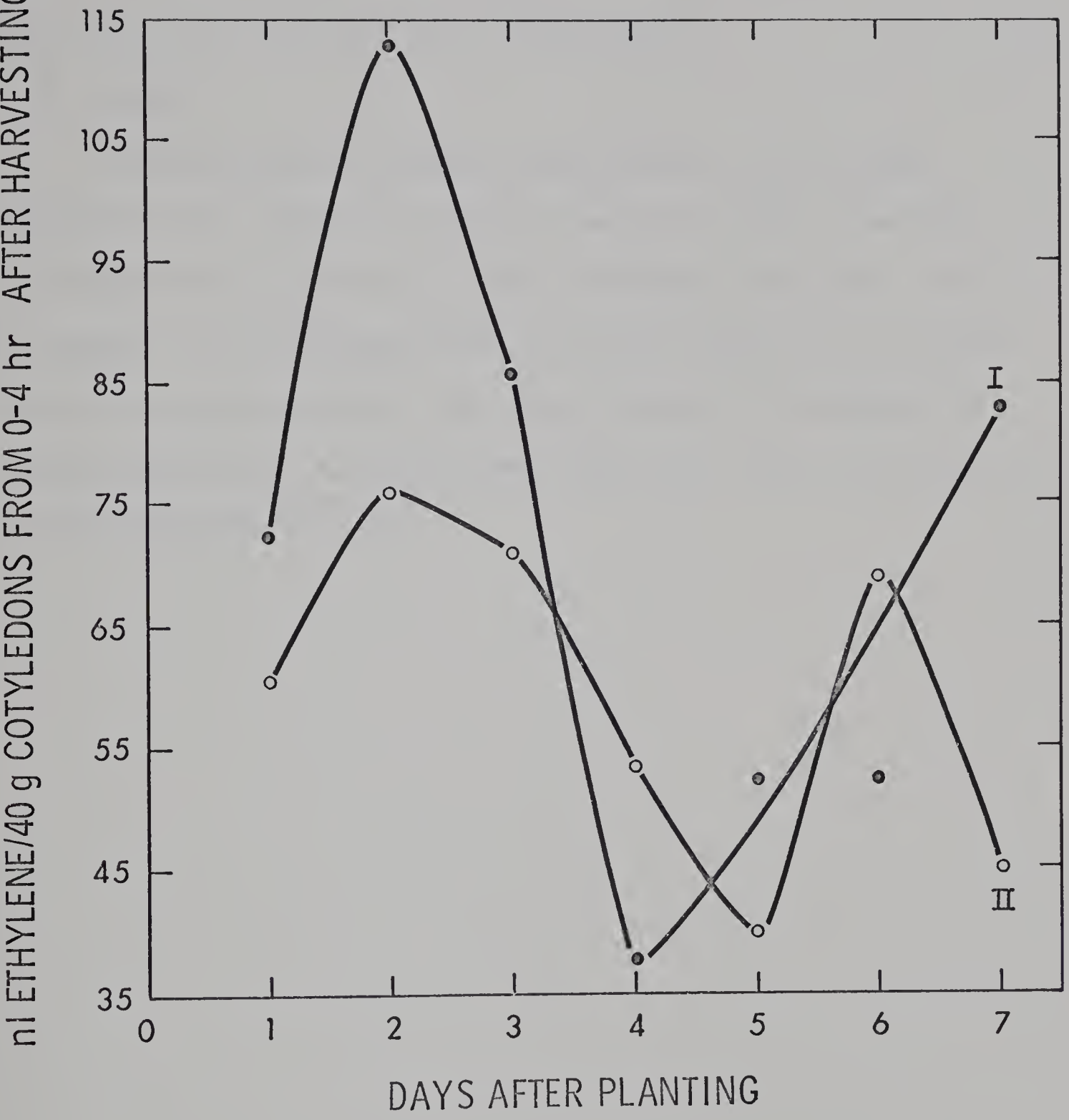
Section A: Association of Ethylene Evolution with Aging of Bean Tissues

1) Cotyledons

Figure 3 shows ethylene evolution from bean cotyledons harvested at different ages. Although the actual amount of ethylene evolved at a certain age was different between the two batches of beans, the patterns of the ethylene evolution with ages of the cotyledons were similar i.e. peaks were observed at day 2 and day 6 (curve II) or a little later (curve I) [for the use of terms day 2, day 6 ...etc. see p. 16]. Stinson (42) obtained two peaks at day 2 and day 6 from bean cotyledons germinated under the same growth conditions. Thompson (56) observed a rather broad peak between day 6 and day 8, but the growth condition was slightly different in that seeds were planted in California soil mix or Perlite R covered with wet tissue and germinated at 24°.

From fig. 3 the two day old cotyledons would seem to be most suitable for use as the source of tissues for enzyme preparations (on the assumption that ethylene producing enzymes would be most active at this stage). Stinson (42), however, found the 32,000 g fraction from the three day old cotyledons to evolve ethylene more actively in the presence of ATP. Since the curve of ethylene evolution with ages of bean cotyledons in the present study was

Figure 3. Ethylene production of whole cotyledons of etiolated beans harvested at times indicated.

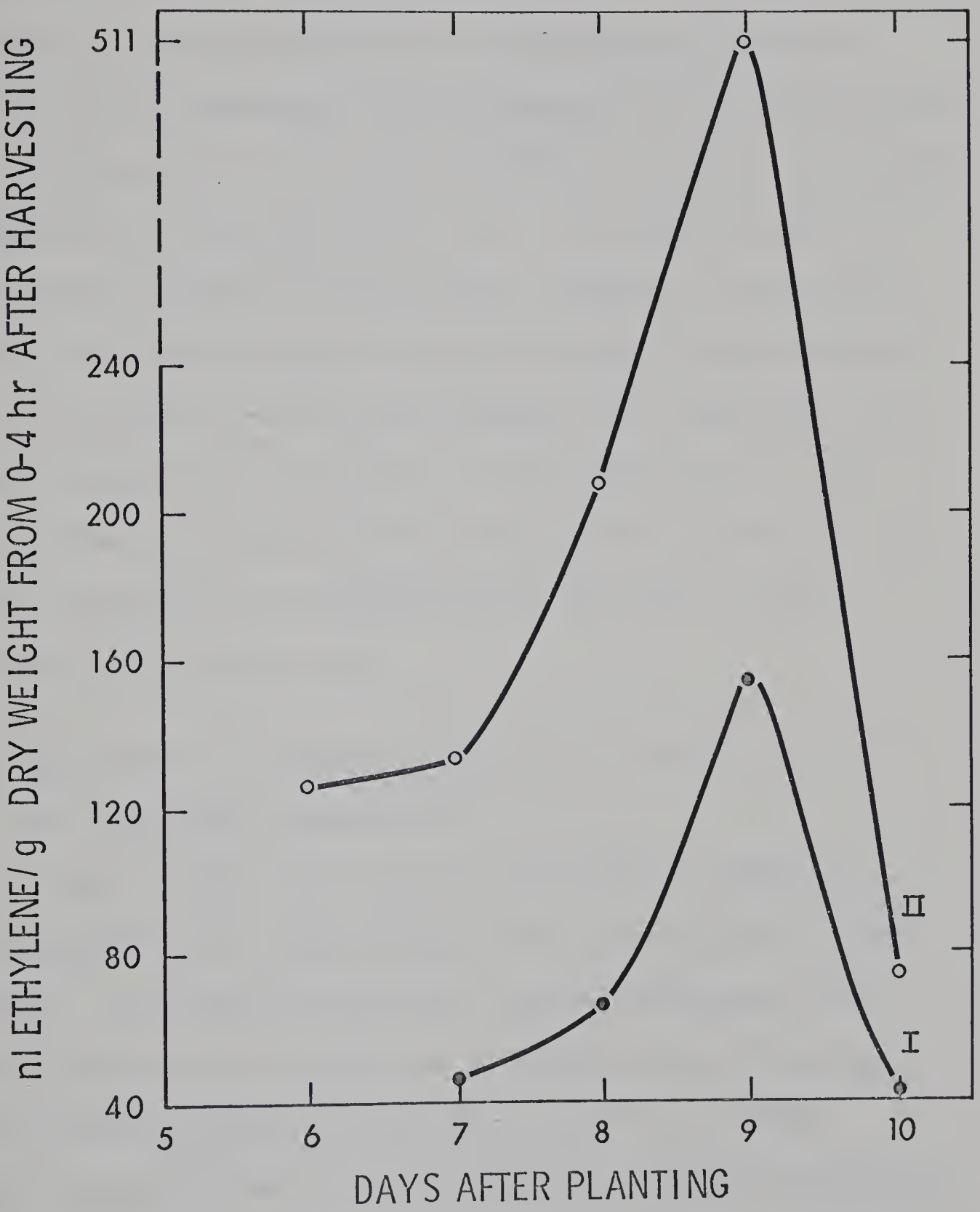

nl ETHYLENE/40 g COTYLEDONS FROM 0-4 hr AFTER HARVESTING
115
105
95
85
75
65
55
45
35
0
1
2
3
4
5
6
7
DAYS AFTER PLANTING
I
II

comparable to that of Stinson's, it was decided to isolate the enzyme system from the three day old cotyledons.

2) Leaves

Maximal ethylene evolution was observed at day 9 after planting (fig. 4). Knight (2) observed two peaks at day 7 and day 10 from bean plants grown under similar conditions except that seeds were planted in soil-peat-sand (3:2:1) mixture instead of in vermiculite as in the present study. For tracer studies of the metabolism of labeled methionine in detached bean leaves, the leaves harvested at day 9 after planting were used.

Figure 4. Ethylene production of bean leaves harvested at times indicated.


nl ETHYLENE/g DRY WEIGHT FROM 0-4 hr AFTER HARVESTING
511
240
200
160
120
80
40
5
6
7
8
9
10
DAYS AFTER PLANTING
II
I

Section B: In vitro Synthesis of Ethylene from β-alanine

Earlier work by Meheriuk on the effect of various substrates and cofactors on ethylene production of the 35,000 g fraction of tomatoes (13), and subsequent work by Thompson (14) on identification of the intermediates for conversion of [^{14}C-2]-β-alanine to ethylene had suggested the involvement of at least four enzymes (see p. 10) in the ethylene synthesis from β-alanine. Recently, Stinson (43) identified and characterized the enzyme β-alanine aminotransferase as far as the identity of the amino acceptor, the requirement for pyridoxal phosphate and the product of the transamination. The activity of the last enzyme for the conversion of acrylate to ethylene, presumably by decarboxylation of acrylate, has been identified in the present study.

1) Stimulation of ethylene production by β-alanine

a) Freeze-thaw preparations

Table 2 shows the stimulation of ethylene synthesis by β-alanine added to the freeze-thawed 32,000 g preparations of bean cotyledons. The ethylene evolved from flasks containing no acid mixture (β-alanine, malonic acid and α-ketoglutaric acid) was an indication of the presence in the enzyme preparation of some endogenous compounds that could be utilized for ethylene production, (the presence of 50 mM malonate and 50 mM α-KG in the assay mixture containing no added β-alanine was found by Stinson to inhibit ethylene production from those endogenous compounds (42)).

Table 2

β-alanine stimulation of ethylene synthesis

by freeze-thaw preparations

Experiment No.	nl ethylene (0-4 hr.) No added β-alanine	β-alanine added	% Stimulation	mg. protein/ assay mixture
I	9.0	20.	122	96
II	5.0	28.0	460	85
III	10.0	25.0	150	68

Reaction mixture: All samples: 1.5 mM ATP; 2.0 mM TPP; 1.0 mM $MgSO_4$; 0.5 mM pyridoxal phosphate; 0.40 mM NADH and 0.17 mM CoA made up in 10 mM TES pH 7.2. Where β-alanine was added, its concentration was 50 mM; these flasks also were 50 mM in malonic acid and 50 mM in α-ketoglutaric acid.

For preparation of the enzyme system see p. 21. Experiments I, II and III were done from different batches of bean cotyledons.

Variations in the amount of these endogenous compounds could therefore be a main factor contributing to the observed variation in the percentage of stimulation by added substrate (i.e. a preparation that was almost saturated with endogenous substrate would seem to be less active towards added substrate). Many workers have realized the necessity of removing as much as possible the endogenous substrate, to demonstrate activity of the enzymes (65). In these experiments and later experiments with soluble enzyme preparations, reproducibility of two identical samples from the same preparation was very good. There was no observable difference in the amounts of ethylene that were produced.

b) Soluble enzyme preparations

The effectiveness of many synthetic detergents in disrupting a membrane bound structure has made their use become more general in the work with particulate enzymes. As well as their usefulness, many adverse effects exerted by these detergents, primarily through their ionic properties, have been observed. In this respect, nonionic detergents would seem to be more advantageous (66). For the release of the ethylene synthesizing enzyme system from β-alanine, a nonionic detergent Triton X-100 was used.

Experiments were done to determine an appropriate length of treatment/the 32,000 g preparation with the Triton buffer. It was found that treatment for one hour with 0.4% Triton buffer followed with 8 hours of incubation of the sediment obtained after centrifugation of the 0.4% Triton treated sample (see p. 21)

yielded the most satisfactory preparations insofar as the enzyme activities and the length of the whole operation were concerned. With this treatment approximately 33% of the total protein in the 32,000 g fraction was rendered soluble.

Table 3 shows the stimulation by β-alanine of ethylene synthesis by the soluble enzyme preparations obtained as above. The percentage of stimulation by β-alanine was lower in experiment I and II than that obtained from the freeze-thawed preparations. Some advantages of using the soluble enzyme preparations, however, are that the purity of the preparation, with respect to the protein component, is greater (only some proteins were rendered soluble), and that the preparations may be further purified with greater ease.

The ethylene evolution from the system containing no β-alanine was high in these experiments and in the experiments done by Stinson (42). In both cases, the preparations had been extensively dialyzed' by passing the preparations through a column of Sephadex for desalting. This suggests that the "enzyme-bound" compounds convertible to ethylene may have been present. The use of Sephadex G-50 in place of G-25 as used by Stinson (42) was intended to increase the efficiency for desalting as well as to remove smaller protein molecules whose molecular weights are less than 30,000 (fractionation range of G-25 is 1,000 - 5,000; but of G-50 is 1,500-30,000 (67)). These small protein molecules include some proteases (68, 69) that might exert their proteolytic activities on the enzyme of interest. (Preliminary work showed detectable proteolytic enzyme activity in the lyophilized 32,000 g preparation).

Table 3

β-alanine stimulation of ethylene synthesis

by soluble enzyme preparations

Experiment No.	nl ethylene (0-4 hr.) No added β-alanine	β- alanine added	% Stimulation	mg. protein/ assay mixture
I	8.5	15.0	77	54
II	4.5	8.8	95	46
III	6.3	13.6	116	46

Reaction mixture: All samples: 1.5 mM ATP; 2.0 mM TPP; 1.0 mM $MgSO_4$; 0.5 mM pyridoxal phosphate; 0.40 mM NADH and 0.17 mM CoA made up in 10 mM TES pH 7.2. Where β-alanine was added its concentration was 50 mM; these flasks also were 50 mM in malonic acid and 50 mM in α-ketoglutaric acid.

For preparation of the soluble enzymes see p. 21. Experiments I, II and III were done from different batches of bean cotyledons.

2) Stimulation of the ethylene synthesis by acrylate

The results obtained in (1) showed that the enzyme system for ethylene synthesis from β-alanine can be prepared in a soluble fraction by treatment with Triton X-100. Since the main object of this research project was to purify these soluble enzyme preparations further, with a special emphasis on the last enzyme of the β-alanine pathway (see p. 11), it would be appropriate to first of all establish the conditions under which the activity of the preparations towards added acrylate could be detected.

a) Effect of dithiothreitol (DTT) on ethylene production of the soluble enzyme preparations

The 'protective' effect of dithiothreitol (DTT) at 3.2 mM and 9.7 mM on the ethylene biosynthetic system, both in the presence and absence of acrylate, was suggested by Stinson (42). Further investigations were made in the present study to determine a suitable concentration of DTT for use in the assay mixture.

As shown in Table 4, addition of DTT in the concentration of 20 mM (approximately twice as high as the highest concentration used by Stinson) caused a decrease by 60% in ethylene production from the enzyme preparation in buffer in experimentI, and by 53% in experiment II. In the presence of all cofactors, however, the decrease was only 40% in experiment I and in experiment II there was a stimulation by 300%. It should be noted that the amount of protein was twice as high in experiment II. The effect of cofactors in stabilizing the active sites on enzyme molecules has been known

Table 4

Effect of DTT on ethylene synthesis from endogenous substrate by the soluble enzyme preparations

Experiment No.	Assay mixture	nl ethylene (0-4 hr.)	Ethane* (0-4 hr.)	mg. protein/ assay mixture
I	Enzyme preparation in 50 mM TES buffer	7.5	0	
	Enzyme preparation in 50 mM TES buffer + 20 mM DTT	3.0	7.9	44.0
	Enzyme preparation in 50 mM TES buffer + 20 mM DTT + all cofactors	4.5	0	
II	Enzyme preparation in 50 mM TES buffer	3.9	0	
	Enzyme preparation in 50 mM TES buffer + 20 mM DTT	1.5	40.4	75.0
	Enzyme preparation in 50 mM TES buffer + 20 mM DTT + all cofactors	16.5	30.4	
III	20 mM DTT + all cofactors	1.0	0	0

Cofactors: 1.5 mM ATP; 2.0 mM TPP; 1.0 mM $MgSO_4$; 0.17 mM CoA, pH 7.2. All preparations in experiment I were the same but differed from those in experiment II.

* Ethane was measured as peak heights in inches

for a long time but the decrease in ethylene production when DTT was added could be because of the strong reducing properties of DTT (redox potential is -0.332 v. at pH 7.0 (70) i.e. about the same as that of NADH). Its presence in such a high concentration may cause rupturing of disulfide bonds that were necessary for the right conformation of the enzyme molecules. The probability of such occurring would seem to be greater in the presence of lower amounts of protein. Therefore the concentration of DTT to be added to the assay mixture should be so selected that its net effect on the enzyme preparations is only to preserve its activity.

The results in Table 5 show that DTT added in the concentrations between 6.5 mM and 58.4 mM to the assay mixture containing 60 mg protein did not cause any inhibition of ethylene synthesis from endogenous substrates. With preparation number I when ethylene production in the absence of DTT was high, a concentration of DTT above 19.5 mM was required for greater utilization of endogenous substrates. Where the endogenous activity was low, such as that obtained from preparation II, a large increase in ethylene production had already been obtained at a lower concentration of DTT (6.5 mM).

Preparation number I and II in Table 4 and preparation I in Table 5 evolved ethane quite significantly in the presence of DTT. All the analyses of gas evolved from whole bean cotyledons showed no ethane. The production of ethane from an *in vitro* system prepared from tissues that do not normally produce the gas

Table 5

Effect of DTT at various concentrations on ethylene synthesis by preparations containing 60 mg. protein

Preparation No.	Concentration of DTT (mM)	nl ethylene (0-4 hr.)	Ethane* (0-4 hr.)
I	0	7.0	0
	19.5	8.0	10.9
	58.4	13.5	33.2
II	0	1.3	0
	6.5	2.7	0
	38.9	3.0	0

Reaction Mixture: 1.5 mM ATP; 2.0 mM TPP; 1.0 mM $MgSO_4$; 0.17 mM CoA; 50 mM TES buffer pH 7.2.

*Ethane was measured as peak heights in inches.

had been observed (see eg. Meigh (7); Lieberman and Mapson (71)). Abeles and Rubinstein (72) found that compounds containing sulfhydryl groups, thioglycolic acid; thioglycerol; cysteine; reduced glutathione and coenzyme A, inhibited ethylene production from cell-free extracts of etiolated pea seedlings. In addition to these compounds was ascorbic acid. In their presence the preparations evolved ethane in quantities equivalent to the amounts of ethylene normally produced.

It is not possible to conclude from the data presented in Tables 4 and 5 the role that DTT may have in the production of ethane. It would be necessary, however, to keep this production of ethane to a minimum. The concentration of DTT at 6.5 mM that did not stimulate ethane production from preparation II in Table 5 would seem to be most suitable for preparations having low endogenous activity. From preparations having high endogenous activity, such as preparation I, Table 5, which showed a linear relationship between peak heights of ethane and concentration of DTT, the ethane evolved in the presence of 6.5 mM DTT would be equivalent to a peak height of 3.5 inches. With such preparations it was not possible by DTT addition to obtain a significant increase in ethylene evolution without the production of relatively large amounts of ethane. Since this was regarded as an artifact, 6.5 mM DTT was chosen as a standard amount of DTT to be added.

b) Effect of acrylate concentration

The DTT dependence of the effect of acrylate concentration on ethylene synthesis by soluble enzyme preparations was observed by Stinson (42). In his study, as the concentration of DTT was increased, a higher concentration of acrylate could be utilized by the enzyme preparations. In none of those cases, however, could the concentration be increased above 0.5 mM. (After a certain concentration of acrylate at a specified concentration of DTT had been reached, the stimulatory effect by acrylate began to decrease gradually until it reached zero). The present study shows an increase in ethylene synthesis with increase in concentration of acrylate (at 6.5 mM DTT) up to 1.0 mM (preparation I, Table 6). Since there was no stimulation by acrylate at 2.0 mM, acrylate at 1.0 mM was chosen to add to the reaction mixture for enzyme assay.

In terms of the percentage conversion of acrylate to ethylene, a simple calculation could perhaps be made at this point, although it would not necessarily be accurate (only the radioactive ethylene converted from radioactive acrylate would give almost a true percentage of conversion. Radioactive acrylate, however, is not commercially available and attempts to synthesize the compound from β-alanine gave a yield of about 12%, of a mixture of acrylic and an unidentified compound as shown by separation on TLC. The calculation is based on the assumption that the stoichiometry of the conversion of acrylate to ethylene is 1:1 acrylic acid, and for simplicity, only the initial concentration of acrylic acid in the reaction mixture will be used.

Table 6

Effect of acrylate concentration on ethylene synthesis by the soluble enzyme preparations

Preparation No.	Concentration of Acrylate (mM)	nl ethylene (0-4 hr.)	mg. protein/ assay mixture
I	0	2.5	
	0.5	9.0	66
	1.0	14.5	
II	0	10.2	
	1.0	26.5	65
	2.0	10.2	

Reaction mixture: 1:5 mM ATP; 2.0 mM TPP; 1.0 mM $MgSO_4$; 0.17 mM CoA; 6.5 mM DTT; 50 mM TES buffer pH 7.2
In the absence of enzyme preparation, only trace amount of ethylene was produced from acrylate and cofactors.

Calculation:

pH of the assay medium = 7.25

pK_a of acrylic acid = 4.25

Concentration of acrylate = 1.0 mM

From Henderson-Hasselbach equation:

$$pH = pK_a + \log \frac{[Salt]}{[Acid]}$$

For this assay system, $pH = pK_a + \log \frac{[Acrylate]}{[Acrylic\ acid]}$

$$7.25 = 4.25 + \log \frac{[Acrylate]}{[Acrylic\ acid]}$$

$$\log \frac{[Acrylate]}{[Acrylic\ acid]} = 3.0$$

$$\frac{[Acrylate]}{[Acrylic\ acid]} = 1000$$

$$[Acrylic\ acid] = 1 \times 10^{-3}\ mM = 10\ \mu M$$

At standard temperature and pressure (0°, 760 mm) 1 nmole acrylate should give rise to 22.4 nl ethylene.

In the reaction mixture, 0.015 µmole (= 15 nmole) acrylic acid was present.

Theoretical amount of ethylene that should be given off

= 22.4 x 15 nl

= 336 nl or 370 nl (27°; 760 mm)

With the most active preparation in Table 6 (preparation II), the amount of ethylene given off within the first 4 hours of the

reaction = 16 nl

$$\% \text{ of conversion of added acrylate to ethylene} = \frac{16}{370} \times 100$$

$$= 4.3\%$$

In such an assay system as that for ethylene synthesis from acrylate where the substrate is very reactive, the actual concentration of acrylate in an assay mixture may be different from the calculated concentration. This is the result of the compound slowly undergoing polymerization in the presence of air. Another factor that might contribute to the change in concentration of utilizable acrylate is the reaction between the sulfhydryl groups of the protein molecules and the acrylate. A reaction that could occur is :

$$E - SH + CH_2 = CH.COOH \longrightarrow E\text{-}S.CH_2\text{-}CH_2\text{-}COOH$$

The probability of such occurrences would be greater in the presence of acrylate at higher concentrations. It is thus conceivable that in reaction mixtures to which higher concentrations of acrylate were added, the actual amounts of the acid present could be smaller.

c) Effect of enzyme concentration

Another complication that was encountered during the investigations of enzyme activity towards acrylate is the variation in enzyme activity with the amount of protein in the reaction mixture. From the same preparation, increase in the amount of protein beyond a certain level did not result in an increase in the absolute amount of ethylene produced (see preparation no. 1,

Table 7). For assessment of the effects of enzyme concentration on ethylene synthesis from acrylate, the results obtained from preparation no. I will be discussed with the results from preparation no. III, since not sufficient data was available for each preparation. (It was not until later in this work that large scale enzyme preparations with satisfactory stability became feasible). Another reason is that the activity of preparation no. I is quite comparable to that of no. III. (Assuming a linear relationship between amount of ethylene produced in nl and the amount of protein of the preparation in mg., the calculated amount of ethylene synthesized by 46.0 mg protein of preparation no. I would be 5 nl. This was exactly obtained from 56 mg. protein of preparation no. III.) With this assumption, it can be seen that the ethylene production was linearly increased with the increase in amount of protein of the preparation up to 72 mg. Beyond this, it is not conclusive from the data as to how the effect of enzyme concentration might be. It seems, however, that no further increase in ethylene production was obtained up to 83 mg protein (preparation no. II, evolved equal amounts of ethylene from 63 mg. and 83 mg.) and that there was a decrease in ethylene production at 103 mg. protein. Indeed, many experiments showed that an increase in amount of protein above 85 mg./assay mixture always produced a low amount of ethylene.

The decrease in ethylene synthesis as a result of having a high concentration of protein in the assay mixture might be explained by: (1) presence of other enzymes that can utilize acrylate, (2) presence of inhibitors for the conversion of acrylate

Table 7

Effect of enzyme concentration

on ethylene synthesis from acrylate

Preparation No.	Concentration of protein (mg./assay mixture)	nl ethylene (0-4 hr.)
I	0	2.7
	51.5	5.8
	103.0	3.3
II	64	16.5
	83	16.5
III	46	5.0
	72	9.0

Reaction mixture: 1.5 mM ATP; 2.0 mM TPP; 1.0 mM $MgSO_4$; 0.17 mM CoA; 6.5 mM DTT; 1.0 mM acrylate; 50 mM TES buffer pH 7.2.

The amounts of ethylene were obtained after subtraction of ethylene from the blanks containing enzyme and cofactors.

to ethylene.

At lower levels of the protein, the activities of those enzymes in (1) and/or the inhibitory effect of the compounds in (2) may not be so significant. At higher levels of protein, however, their effect would become more prominent.

In general, the level of protein in each assay mixture was kept within the range between 64 to 83 mg. (i.e. 6.4 - 8.3 mg./ml). It was found earlier (p. 44) that this range of protein was suitable for use in the assay mixture containing 6.5 mM DTT.

3) Assessment of other possible sources of ethylene

a) Non-enzymic systems

A few experiments were done to see if boiling of the soluble enzyme preparations would have any effect on the ethylene producing activity of the preparations. The results obtained showed that the preparations that were boiled for 10 minutes and left to cool down to 27° (assay temperature) synthesized no ethylene from acrylate.

b) Bacterial contanimants

A number of microorganisms are known to produce ethylene (1). Since all the experiments in the present study were carried out under nonsterile conditions, it was necessary to see if the bacterial contaminants of the preparations produced ethylene.

After the ethylene producing activity of the soluble enzyme preparation had been assayed for, the assay mixture was

plated out on nutrient agar medium by a serial dilution technique (each dilution was done in triplicate) (73). The plates were incubated at 37° for 24 hours and the number of bacterial colonies was counted. From the number of colonies and dilution factor for each plate, the number of bacterial cells per reaction flask was calculated. This was found to be 5.505×10^6 bacterial cells. No mold appeared on these plates.

Bacteria were picked up from each type of colony and inoculated under sterile conditions into nutrient broth (1000 ml.). They were left to grow at 37°C for two days, then harvested. The bacterial suspension was centrifuged at 32,000 g for 15 minutes, at 0° - 4° in an International Refrigerated Centrifuge Model B-20. The cells were then suspended in 10 mM TES buffer pH 7.2 and assayed for ethylene production from acrylate (in a complete assay medium p. 26). No detectable amount of ethylene was obtained.

After 0-4 hours of assay, a small aliquot was taken and the number of bacterial cells was determined as before. From the most dilute sample, the colonies were too crowded on the plate to count. The plate prepared from enzyme preparation at the same dilution was found to have 367 colonies present.

From these experiments, it can be concluded that although the soluble enzyme preparations were contaminated with bacteria, these bacteria did not produce ethylene from acrylate under the assay conditions chosen. The disadvantage of this method of studying contamination by bacteria is that the nutrient-broth agar

medium was assumed to be suitable for growth of all kinds of micro-organisms. In fact the range of organisms grown on such medium does not represent all bacteria that were present in the preparation and the assay mixture, but rather, it represents the types that tolerate these particular growth conditions best.

4) Variation in activity of the soluble enzyme preparations for acrylate, and their activity on storage.

The activity of the enzyme from different preparations varied in the range between 0.10 - 0.25 nl ethylene produced / mg. protein. Since the preparation of the enzyme involved isolation of subcellular particles and treatment with Triton X-100 to render the enzyme system soluble, uniformity in activity could not be expected in view of current limitations in our knowledge of these techniques.

The soluble enzyme preparations, after lyophilization, could be stored under vacuum at temperature of the deep freeze (approximately -15°) for one week with no loss in activity. Activity after a longer storage period was not studied.

Section C: Investigations towards Purification of the Ethylene Synthesizing Enzyme System Stimulated by Acrylate

With the discovery that the soluble enzyme preparations catalyzed ethylene synthesis from acrylate, it was desirable that the preparations be purified further. Three approaches were taken, which were:

1) fractionation with ammonium sulphate,
2) heat-denaturation of the heat sensitive proteins,
3) fractionation by gel chromatography.

1) Fractionation with ammonium sulphate

The salt fractionation technique has many advantages over some other methods of enzyme purification. It is easy to carry out without expensive materials or equipment. Ammonium sulphate has been used frequently for the purification purposes as well as for its protective action on enzymes (74). A disadvantage of ammonium sulphate, however, is that enzymes do not come down within fixed limits of salt concentration characteristic of each enzyme, but the limits vary with the concentration of the enzyme (74). The slightly acidic character of ammonium sulphate is another disadvantage to its use in enzyme purifications. Control of pH is difficult as the result of a loss of ammonia and, in a strong ammonium sulphate solution, a determination of the pH is not easy because of salt errors and junction potentials (74). It was recommended by Dixon and Webb that the salt should be diluted to less than 0.3 M before pH determination is attempted.

Thompson (56) purified the butanol treated enzyme powders from bean cotyledons by precipitation with ammonium sulphate. The highest ethylene synthesizing activity from β-alanine was found in the fraction that was precipitated by saturation with 65 - 100% ammonium sulphate. In the present study, activity of the enzyme for ethylene synthesis from acrylate was found in all the protein fractions that were precipitated with ammonium sulphate (table 8). Saturation of all the preparations, which were tested, with 30% ammonium sulphate caused cloudiness of the protein solution (presumably by the salting out effect of the salt). Subsequent centrifugation of the suspension at 10,000 g for 10 minutes, however, did not sediment the protein. Saturation with 40% ammonium sulphate yielded a protein suspension that could be sedimented by centrifugation at 10,000 g for 10 minutes, but the amount of the protein so obtained was insufficient to exhibit the ethylene synthesizing activity (if such were present in the fraction). While the 70 - 100% fraction showed a great variation in the enzyme activity, the 40 - 70% fraction always gave substantial amounts of ethylene that were reproducible. Therefore, saturation of the solution of soluble enzyme preparations with 40 - 70% $(NH_4)_2SO_4$ was chosen as a means of purifying the preparations. The purification that was achieved was 2.3 fold (preparation III, table 8). The absolute amount of ethylene produced from the purified fractions was higher than that of the original soluble enzyme preparations before subjected to $(NH_4)_2SO_4$. Three possible effects of $(NH_4)_2SO_4$ might be involved:

Table 8

Ethylene production of fractions precipitated from the soluble protein preparations by saturation with ammonium sulphate

Preparation No.	Treatment	nl ethylene (0-4 hr.)	mg protein	nl ethylene/ mg protein
I	0-50% $(NH_4)_2SO_4$	14.5	64	0.28
	50-70% $(NH_4)_2SO_4$	10.0	46	0.22
	70-100% $(NH_4)_2SO_4$	8.7	39	0.22
II	40-70% $(NH_4)_2SO_4$	28.5	84	0.34
	70-100% $(NH_4)_2SO_4$	5.0	32	0.16
III	Purification achieved			
	a) soluble enzyme preparation	26.5	51	0.52
	b) 40-70% $(NH_4)_2SO_4$	44.5	35	1.2

Reaction mixture: 1.5 mMATP; 2.0 mM TPP: 1.0 mM $MgSO_4$; 0.17 mM CoA; 6.5 mM DTT; 1.0 mM sodium acrylate; 50 mM TES buffer pH 7.2. Assay temperature was 27°.

i) a stimulatory effect of $(NH_4)_2SO_4$ on the enzyme,

ii) a removal of 'unknown factors' that might be repressing the activity of the enzyme in the original soluble enzyme preparations,

iii) a possible stabilizing effect on the enzyme by $(NH_4)_2SO_4$.

Addition of $(NH_2)SO_4$ to the assay mixture to give a final concentration of 2% was found to cause a slight increase (by 10%) in the absolute amount of ethylene produced. A final concentration of 4% in ammonium sulphate caused neither a stimulation nor an inhibition. No further work was done, however, to determine the effect of $(NH_4)_2SO_4$ at other concentrations. (The amount that could be remaining in the protein fraction was not estimated.)

2) Heat denaturation of the heat sensitive proteins

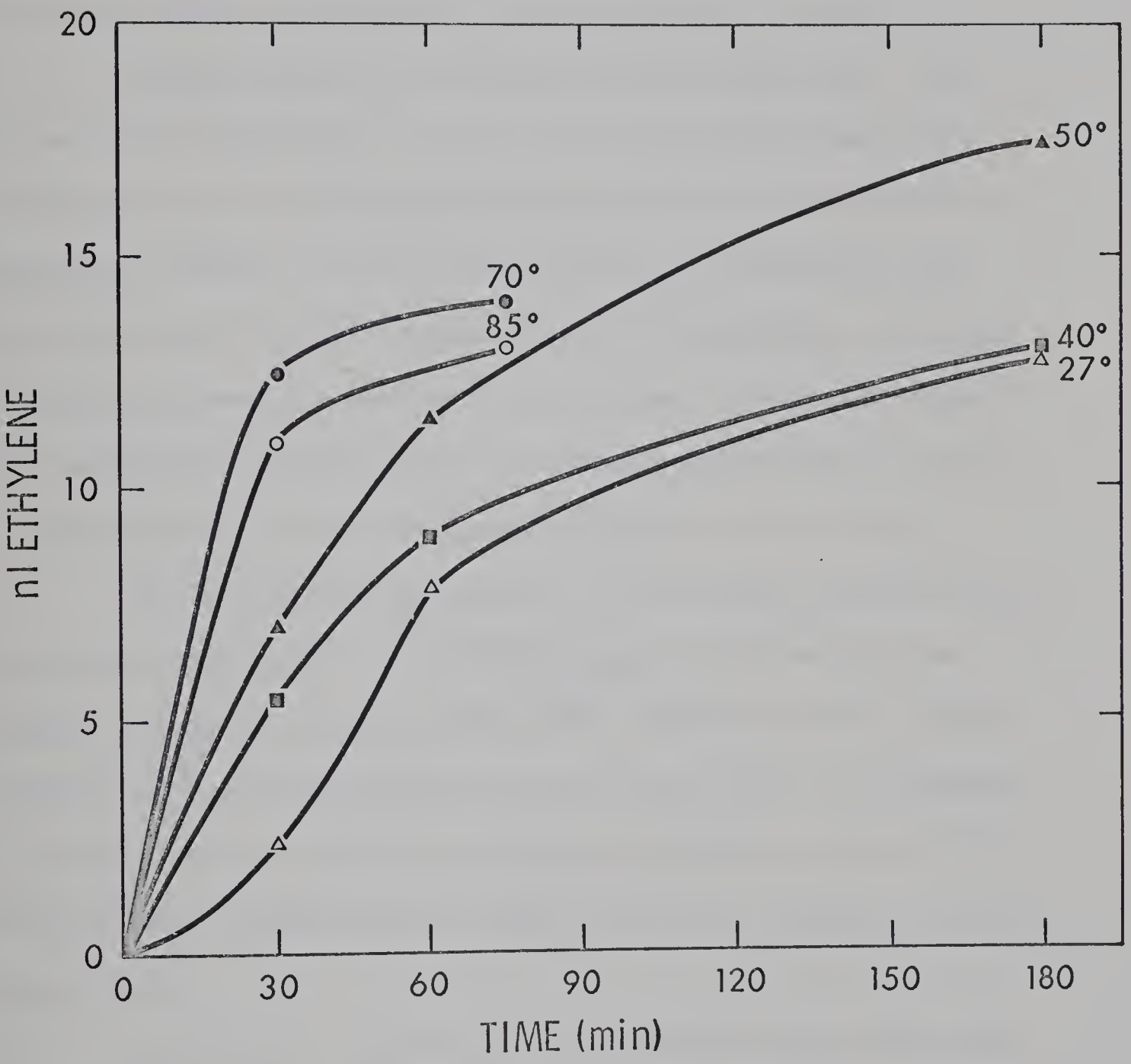
Spencer and Meheriuk (75) reported that the ethylene production from the 35,000 g fraction from a tomato homogenate, when incubated in a complete medium, was higher at 100° than at 25°, over the first two hours. At intermediate temperatures (30° and 40°) there was not much difference in ethylene production from that at 25°. In the present study, the author observed a great amount of ethylene evolved from the preparations, salted out between

40 - 70% saturation with $(NH_4)_2SO_4$, that had been boiled for a few seconds and immediately added to the complete assay mixture. The effect of temperature was, therefore, investigated further to obtain information that might be useful for purification purposes as well as for a possible alternative assay condition for the enzyme preparations.

The preparations that were purified from (1) were heated to 40°, 50°, 70° and 85° by holding test-tubes containing the preparation in a boiling water bath. In all the cases, except the preparation to be heated to 85°, time taken to reach the desired temperatures was less than one minute. Immediately, these preparations were added to the assay flasks containing complete assay mixture held at 27° and ethylene collection was started. The actual assay temperature in each flask therefore was initially a little lower than the temperature of the preparation, and decreased gradually until it reached the temperature of the water-bath (27°; time taken was less than 30 minutes).

When the absolute amount of ethylene produced from each preparation was plotted against time of collection of ethylene, the curves were obtained as shown in figure 5. A lag period of 30 minutes was observed with the preparation that had been held at 27°. With other preparations, the ethylene evolved increased with an increase in temperature up to approximately 70°. A temperature dependence of the velocity of a chemical reaction is a common phenomenon (76). The velocity of an enzymic reaction affected by

Figure 5. Effect of temperature on activity of the enzyme that catalyzes ethylene synthesis from acrylate. Assay medium was 1.5 mM ATP; 2.0 mM TPP; 1.0 mM $MgSO_4$; 0.17 mM CoA; 6.5 mM DTT; 1,0 mM sodium acrylate; 50 mM TES buffer pH 7.2. Amount of protein in each assay mixture was 68 mg. 0 -30 min. (or less) was the period during which the preparations were cooling down to 27°. From 30 min. on, temperature in all the assay flasks was 27°.


20
15
10
5
0
nl ETHYLENE
50°
70°
85°
40°
27°
0
30
60
90
120
150
180
TIME (min)

temperatures, however, is a net result of the temperature-dependent velocity of the reaction and the temperature-dependent denaturation of the enzyme proteins. From figure 5, the latter effect seems to be more prominent on the preparation heated to 70° and 85°.

Further evolution of ethylene at 27° between 30 - 60 minutes (figure 5) showed a similar activity of the preparations previously heated to 40° and 50°, to that of the preparation not subjected to heating. During this period the preparations that were heated at 70° and 85° evolved very little ethylene, although in the period immediately after heat treatment, these two preparations evolved more ethylene than any other preparations in figure 5. From the results, some conclusions may be made as follows:

i) the ethylene production of the protein fractions that were salted out between 40 - 70% $(NH_4)_2SO_4$ was increased by an increase in temperature up to about 85°. At 70° and 85°, however, the effect of heat denaturation of the enzyme protein was dominant. As a result, after an increase in ethylene evolution over a brief period of time, preparations no longer catalyzed ethylene synthesis from acrylate.

ii) as far as stability of the cofactors was concerned, it could be concluded that no changes in their properties were brought about by heating in the reaction mixture to 50°, since the ethylene production from the two flasks containing enzyme preparation previously heated at 40° or 50°, was the same as that from the flask containing the preparation that had been kept at 27°.

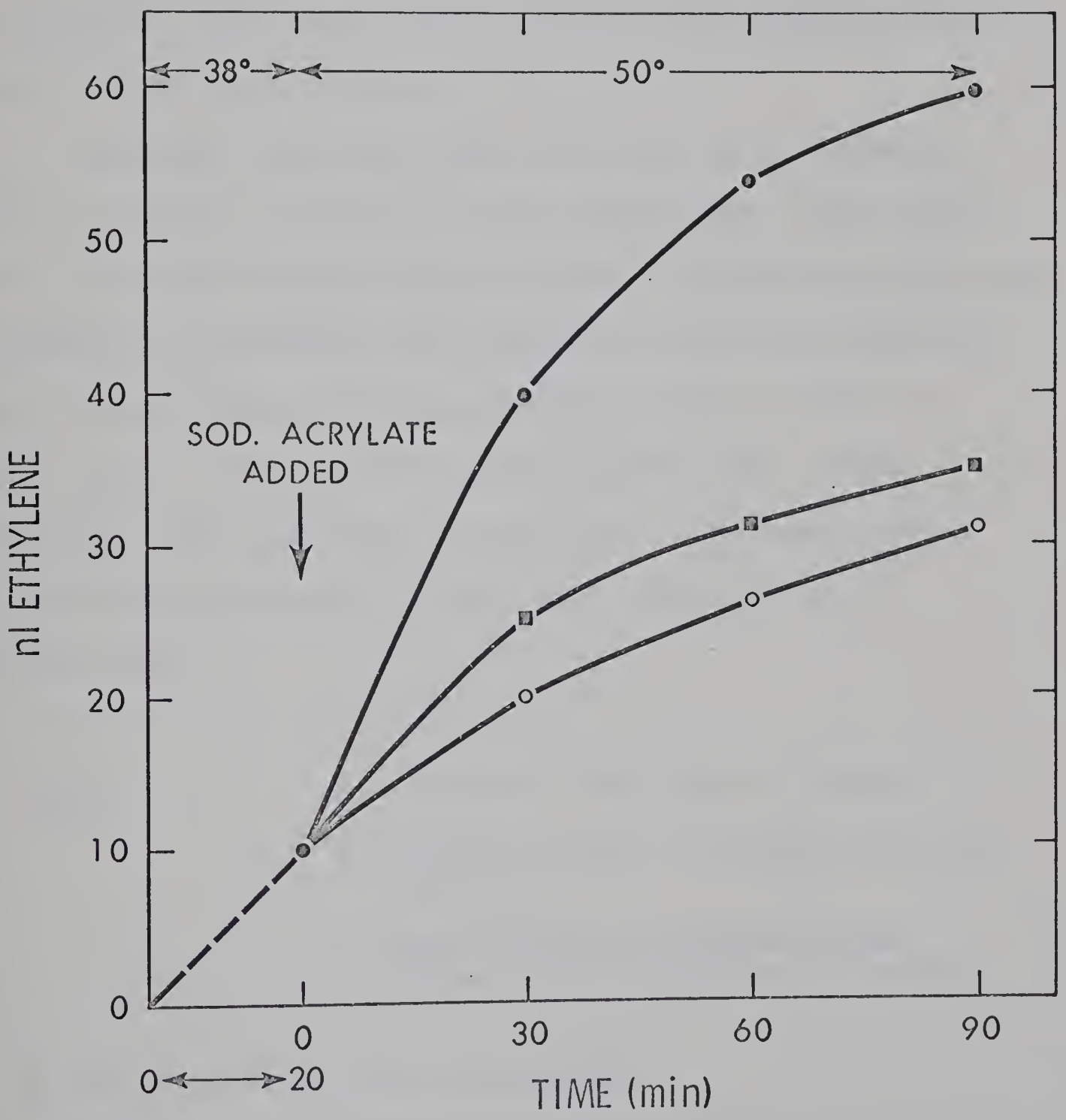
The same reasoning is also applicable to the properties of the enzyme in this range of temperatures.

iii) the lag period of 30 minutes that was obtained from the enzyme preparation kept at 27° could perhaps be explained as a period during which reconstitution of the enzyme-cofactors complex occurred. A reconstitution period of 20 - 30 minutes at room temperature for the yeast pyruvate decarboxylase apoenzyme-cofactors complex has been allowed for by Ullrich (77) before addition of pyruvate and other reagents. Morey and Juni (78) found the reconstitution to be a relatively slow process and that the rate of reconstitution was a function of temperature, the concentration of TPP and divalent cations.

When the enzyme preparation was incubated with all the cofactors at 38° for 20 minutes prior to addition of 1.0 mM acrylate and an increase in temperature to 50°, a significant stimulation of ethylene production was obtained (figure 6). (This was repeated once.) Since the stimulation of ethylene production from this flask was greater than for those from the other two flasks containing no acrylate, it can be concluded that such stimulation was the result of adding acrylate to the assay mixture. (All flasks were subjected to the same temperature regime.)

In all the experiments at higher temperatures a considerable amount of ethane was evolved (much greater than ethylene). The preparations that were heated for a few seconds at 50°, after cooling down, also evolved a large amount of ethane at 27°. (The ethane

Figure 6. Time curve study of ethylene synthesis from acrylate at 50°. (0-0) soluble enzyme preparation in TES buffer; (■-■) enzyme preparation and all the cofactors; (o-o) enzyme preparation and all cofactors with sodium acrylate added after 20 min. of incubation of the enzyme with the cofactors at 38°.


38°
50°
60
50
40
30
20
10
0
ml ETHYLENE
SOD. ACRYLATE
ADDED
0
30
60
90
0
0←→20
TIME (min)

production stimulated by DTT has already been discussed (p. 44). Since there is no information available at present for the cause of this ethane production *in vitro*, the knowledge gained from the study of temperature effect on ethylene production from acrylate should perhaps, at this point, not be applied to the preparations as a means of further purification.

One other factor that might contribute to the 'apparent' increase in ethylene evolution from the assay system at high temperatures is the solubility of various gases as a function of temperature. The temperature dependence of the solubility coefficient values for oxygen, carbon dioxide, ethylene and ethane in water is shown in table 9. In all the cases the K values increase with increase in temperatures, hence a decrease in solubility. From these values, the solubility of each gas at a particular temperature can be calculated from:

$$K = P/X$$

where

K = solubility coefficient of the gas

P = partial pressure of the gas above the solution

X = molar fraction of the gas in the solution (i.e. solubility of the gas)

3) Fractionation by gel chromatography

Four grades of Sephadex were developed for fractionation of proteins according to their molecular weights. These are

Table 9

Solubility coefficient of oxygen, carbon dioxide, ethylene and ethane in water at various temperatures (79)

	$K \times 10^{-7}$ at					
Gas	0°	10°	20°	30°	40°	50°
Oxygen	1.91	2.48	2.95	3.52	4.14	4.50
Carbon dioxide	0.555	0.788	0.108	0.139	0.173	0.217
Ethylene	0.419	0.584	0.774	1.10	1.20	-
Ethane	0.9547	1.4386	1.9978	2.5976	-	-

G-75 has a fractionation range for proteins with molecular weights between		3,000 and 70,000
G-100	"	4,000 and 150,000
G-150	"	5,000 and 400,000
G-200	"	5,000 and 800,000

(data are given by the manufacturer ref. 67)

It is obvious then that the technique would not be useful to separate the proteins in a mixture if they have approximately the same molecular weights. Some factors to be considered in the application of the techniques to purification of enzymes are:

a) Sample size and viscosity of the sample

For a good separation, the starting zone (i.e. sample zone) should be made narrow by applying a small sample. The Pharmacia Fine Chemical Company recommends using a sample volume of 1 - 4% of the total column volume (67) for Sephadex G-100, G-150, and G-200. When approximately 8.0 ml. soluble protein in TES buffer was layered on the gel packed in a K25/45 column (dimension of 2.5 x 45 cm, giving the total volume of 220.0 ml.), however, the sample band was found to be rather broad. As it moved down the column, broadening of the protein band became more pronounced. It was necessary then to reduce the sample size and increase the protein concentration of the sample so as to obtain sufficient amount of the enzyme for activity assay. As the result, the viscosity of the sample was increased. It was found that such a viscous

solution caused distortion of the sample zone, and hence poor separation of the protein components. To compromise between the sample size, the concentration of the protein sample, a good separation of the protein components and a detectable activity of the purified fraction, a sample size of 3.0 ml. containing between 50 - 60 mg. protein/ml. was used.

b) Flow rate

The higher numbers of Sephadex have a greater water regain values than the lower ones. A high flow rate exceeding the theoretical maximal flow rate therefore caused collapsing of the gel particles and an irregular pattern of flow through the column. The following is recommended for Sephadex G-100, G-150 and G-200 packed in a column whose dimension is 2.5 x 100 cm (65):

Sephadex G-100; dry particle diameter 40-120 μ; optimal flow rate = 72 ml/hr

Sephadex G-150; 40-120 μ; optimal flow rate = 34 ml/hr

Sephadex G-200; 40-120 μ; optimal flow rate = 18 ml/hr

The flow rate, however, was inversely proportional to the length of the column (65). Therefore for the column whose dimension is 2.5 x 45 cm, used in the present study, the optimal flow rates would be approximately twice as high as those shown above.

In practice, however, it was necessary to operate the G-200 column at the flow rate of 12.0 ml./hr and the G-100 column

at the flow rate of 20.0 ml./hr to obtain satisfactory separations of proteins of known molecular weights (cytochrome C MW 12,400; pepsin MW 35,000; bovine serum albumin MW 66,500 and yeast alcohol dehydrogenase MW 150,000). A very low flow rate such as that used on the G-200 column would not be useful for enzymes that have low stability.

Two grades of Sephadex, the G-200 and the G-100, were tested in the present study.

a) Sephadex G-200

A preliminary run was made with a soluble enzyme preparation in the complete assay medium after the enzyme activity had been assayed. The sample size of 2.0 ml. (4.0 mg. protein/ml) was used. Protein was detected in fraction number 27 and 28 (4 ml/fraction).

Void volume (Vo) for this column as determined by blue dextran (67) was 64.0 ml.

The elution volume (Ve) of fractions number 27 and 28 = 108.0 ml. For G-200; MW is calculated from

$$\log M = 6.698 - 0.987\ (Ve/Vo) \qquad (80)$$

where M = Molecular weight

$$\log M = 6.698 - 0.987\ \frac{(108)}{64}$$

$$= 6.698 - 1.665 = 5.033$$

Molecular weight = $1.079 \times 10^5 = 107,900$

From this column, calculated MW for cytochrome C = $1.507 \times 10^4 = 15,070$ (Theoretical MW = 12,400)

The elution profile of the soluble protein sample (3.0 ml. containing 50 mg. protein/ml. solution) from the Sephadex G-200 column is shown in figure 7.

Although the Sephadex G-200 has the widest fractionation range (see p.65) for proteins whose molecular weights are between 150,000 and lower, the use of G-200 would not have any advantage over the use of G-100. On the contrary, the use of G-100 would speed up the whole process of fractionation of the proteins (because of its higher operating flow rate). Sephadex G-100 was therefore tested.

b) Sephadex G-100

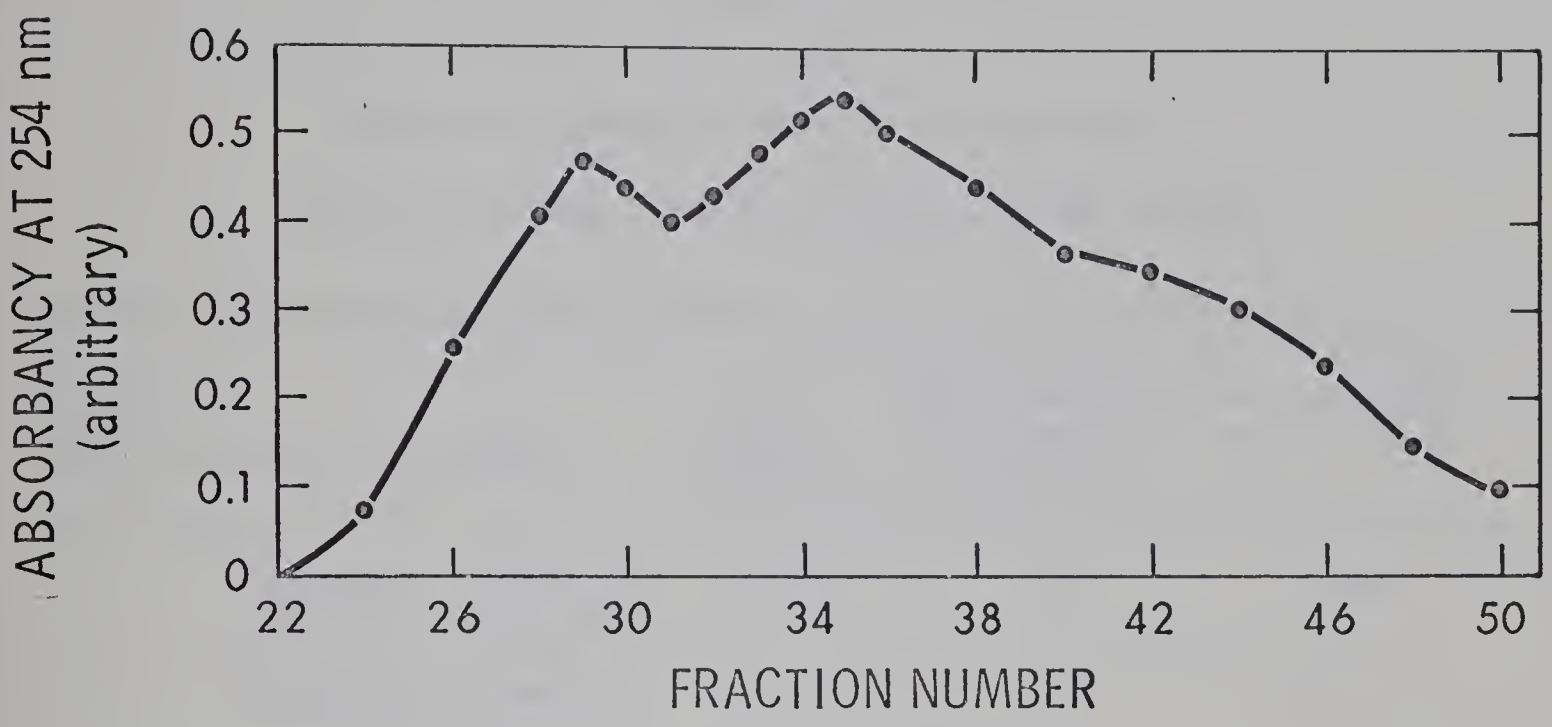
From the G-100 column, a broad peak of noncoloured protein solution was obtained from fractions number 29 to 36. From fraction number 36 on, the solutions were very yellow and absorbed light at 254 nm (wavelength at which absorbancy is detected by the Uvicord). When the broad, noncoloured protein peak was divided into two portions (fraction number 29 to 32, and fraction number 33 to 36), the ethylene synthesizing activity from acrylate was found in the first half of the peak (preparation number 1, table 10). Since the amount of protein of the second half of the peak in the assay mixture was higher than that of the first half of the peak (45 mg. per assay mixture as opposed to 21 mg./assay mixture of the latter), absence of the enzyme activity from this fraction of the protein could not be the result of having insufficient protein in the assay mixture.

Figure 7. Chromatography of soluble enzyme preparation on 2.5 x 45 cm. Sephadex G-200 column. Proteins were eluted with 0.1% Triton in 10 mM TES pH 7.6 at 0°; void volume (Vo) was 64 ml. as measured with Blue Dextran 2000.

Figure 8. Chromatography on 2.5 x 45 cm. Sephadex G-100 column of soluble enzyme preparation. Proteins were eluted with 0.1% Triton in 10 mM TES pH 7.6 at 0°; void volume (Vo) was 63.8 ml as measured with Blue Dextran 2000, elution volume of the enzyme for ethylene synthesis from acrylate was 70.4 ml.

(o—o) = absorbancy at 254 nm

▨ = activity of the enzyme


ABSORBANCY AT 254 nm
(arbitrary)
0.6
0.5
0.4
0.3
0.2
0.1
0
22
26
30
34
38
42
46
50
FRACTION NUMBER

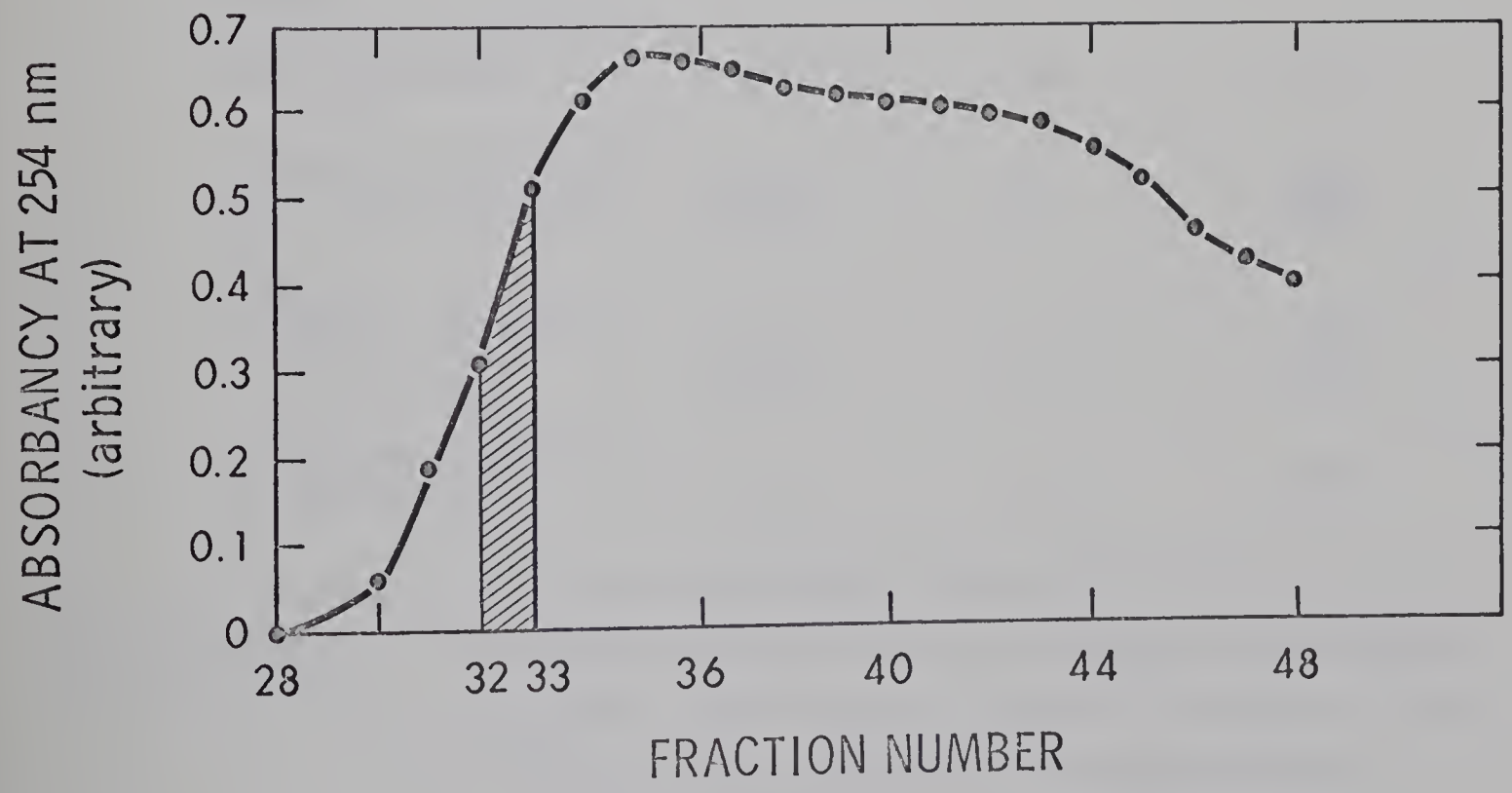

ABSORBANCY AT 254 nm
(arbitrary)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
28
32 33
36
40
44
48
FRACTION NUMBER

Table 10

Ethylene production from various protein fractions eluted from the Sephadex G-100 column

Preparation No.	Fraction Number	nl ethylene (0-4 hr)	Total protein (mg)/combined fraction	nl ethylene/ mg. protein
I	Combined fractions # 29, 30, 31 and 32	12.0	42	0.60
	Combined fraction # 33, 34, 35 and 36	0	45	0
II	(a) Combined fraction # 30, 31 and 32	11.5	40	0.57
	(b) Combined fraction # 29, 30 and 31	0	30	0.0
	Combined fraction # 32, 33, 34 and 35	19.0	67	0.57
	*(c) Combined fraction # 32 and 33	12.0	27	0.50
	Combined fraction # 34 and 35	2.0	22	0.09

Reaction mixture: 1.5 mM ATP; 2.0 mM TPP; 1.0 mM $MgSO_4$; 0.17 mM CoA; 6.5 mM DTT; 1 mM acrylate; 50 mM TES buffer pH 7.2 nl ethylene was obtained after subtraction of ethylene from the blank containing enzyme and cofactors. Assay temperature was 27°.

*Amount of soluble protein was 3/4 as high as that in (b); each combined fraction was assayed in the presence of acrylate only but for subtraction, ethylene from the blank was assumed to be the same as that in (b) since the preparation No. II had a very low endogenous activity.

The first half of the noncoloured protein peak was fractionated further (preparation number 11, table 10) and the enzyme activity was found mainly in fraction numbers 32 and 33 (from the table, it seems that the ethylene synthesizing activity was greater in tube 32 than in tube 33. The use of both fractions together for enzyme assay, however, was to obtain as high as possible an absolute amount of ethylene produced, for the maximum accuracy of the ethylene measurement. The author realized that using the fraction number 32 alone would perhaps give a higher purity of the preparation).

Unlike the purification achieved by fractionation with $(NH_4)_2SO_4$, the purification by gel chromatography on Sephadex G-100 was accomplished by removal of some undesired proteins, leaving the absolute amount of ethylene produced unaltered. One other advantage is that there was a clear cut in activity of the preparation (i.e. activity was concentrated in two protein fractions only, as compared to a widespread of activity in all the fractions that were precipitated by saturation with $(NH_4)_2SO_4$. For these, the use of Sephadex G-100 would be more advantageous.

The elution profile and ethylene synthesizing activity of the fractions is shown in figure 8. The purification that was achieved was shown in table 11.

The elution profiles of the proteins from Sephadex G-200 and G-100 (as shown in figures 7 and 8) do not show sharp separations of the protein components. Apart from a possible explanation based

Table II

Purification by Sephadex G-100 of the enzyme catalyzing ethylene synthesis from acrylate

Procedure	* % yield	nl ethylene/ mg. protein	** Purification
1) 32,000 g for 15' fraction	100	0.11	-
2) Soluble fraction from treatment of (1) with 0.4% Triton X-100 for 1 hr. followed with 0.1% Triton for 8 hr.	33	0.24	Twofold
3) Soluble fraction from (2) after fractionation by Sephadex G-100	3	1.4	Twelve-fold

Reaction mixture: 1.5 mM ATP; 2.0 mM TPP; 1.0 mM $MgSO_4$; 0.17 mM CoA; 6.5 mM DTT ; 50 mM TES buffer pH 7.2: 1.0 mM sodium acrylate. Assay temperature was 27°.

* % yield was worked out as % of original mg. protein in (1)

** Purification worked out as increase in nl ethylene /mg. protein over that of the 32,000 g preparation in (1).

on close molecular sizes of these proteins (p. 65), one could picture these broad peaks as being a result of interactions between surface charges on the protein molecules, or interaction between the molecules by other forces. This effect would tend to be greater in a more concentrated solution such as that of the protein solution used in these experiments. In such cases, the proteins having different molecular weights would emerge from the column as a single peak.

Section D: Studies on Some Properties of the Purified, Acrylate Stimulated Enzyme Preparations for Ethylene Synthesis

For all the experiments in this section, the enzyme preparations purified by gel chromatography on Sephadex G-100 were used.

1) Molecular weight determination

On the assumption that the sequence of elution of proteins from a Sephadex column is determined by molecular size of each protein molecule, the molecular weight of the protein(s) that emerged from the Sephadex G-100 column, in fractions 32 and 33, can be calculated from the following equation (80):

$$\log M = 5.941 - 0.847\ (Ve/Vo)$$

M = molecular weight of the protein

Ve = elution volume of the fraction = 70.4 ml.

Vo = void volume as determined by blue dextran = 63.8 ml.

$$\log M = 5.941 - 0.847\ (70.4/63.8)$$

$$= 5.941 - 0.9402$$

$$= 5.0008$$

Molecular weight = 1.019×10^5

(Molecular weight calculated from fractions that were eluted from G-200 = 1.079×10^5, thus a difference of approximately 5% from that calculated from G-100. Since flow rates through the two columns were different, and the sample size and concentration of the sample used in each column were quite different, i.e., 2.0 ml.

of 4.0 mg. protein/ml in the case of G-200, and 3.0 ml. of 50 - 60 mg. protein/ml in the case of G-100, it is not surprising that such a difference would occur.)

2) Electrophoretic properties of the purified enzyme preparation

In order to determine the purity of the preparation that was eluted from the Sephadex G-100 column and catalyzed ethylene synthesis from acrylate, the protein fraction was analysed by 'gel electrophoresis' in an alkaline and in an acidic medium (pH 7.8 and pH 4.5 respectively). From the alkaline medium, four bands of the blue colour appeared after the gel had been destained with 7% acetic acid. From the acidic medium, however, only three bands were observed. It is probable that one of the protein components has its isoelectric point around 4.5.

For studies on kinetic properties of the enzyme that catalyzes ethylene synthesis from acrylate, and for studies of the mechanism of the reaction, it would be necessary to have a purer enzyme preparation. The results that were obtained from the studies of the electrophoretic properties of the preparations suggest one possible method for further purification of the preparations. This is a fractionation of the proteins by gel electrophoresis in an alkaline medium. As far as the enzyme activity at an alkaline pH is concerned, there has been evidence from a study of Stinson (42) that the ethylene production from β-alanine, catalyzed by the soluble enzyme preparations, at pH 8.0 over 0 - 2 hours was about 80% as high as that at pH 7.2. Over 2 - 4 hours, the ethylene production

at these pHs was almost the same. Since the decrease in enzyme activity at pH 8.0 after the first four hours was only 22%, treatment of the preparation at pH 8.0 for four hours on a gel electrophoresis preparative column would perhaps not cause a significant loss in the activity of the preparation. For such technique, however, a rise in temperature as the result of heat generation in the electrophoretic system could cause denaturation of the protein enzyme. Since the electrophoresis experiments in the present study were done at room temperature, the above suggestion for further purification of the enzyme preparations by gel electrophoresis should be reserved until further work is done to see if electrophoresis at a lower temperature (possibly 0° - 4°), the temperature range that had been selected for all the processes involved in preparations of the enzyme in the present study and other studies (42, 56), would give the same pattern of separation of the protein components.

3) Activity of the preparations towards other 'potential precursors'

The partially purified enzyme preparations that were eluted from the Sephadex G-100 column could catalyze ethylene production from the following substrates (listed in order of increasing ethylene production): L-methionine <β-alanine < propionate < pyruvate < acrylate. (The concentration of each substrate used and the ethylene evolved/mg. protein are shown in table 12.)

The production of ethylene from substrates other than acrylate suggests two possibilities:

Table 12

Ethylene synthesis from various

'potential precursors' by purified enzyme preparations

Substrate	nl ethylene (0 - 4 hr.)	nl ethylene /mg. protein
1 mM acrylate	32.5	1.80
1 mM propionate	27.5	1.40
1 mM pyruvate	21.5	1.66
1 mM L-methionine	trace	trace
50 mM β-alanine + 50 mM malonate and 50 mM α-KG	12.5	1.0

Reaction mixture: 1.5 mM ATP; 2.0 mM TPP; 1.0 mM $MgSO_4$; 0.17 mM CoA; 6.5 mM DTT; 50 mM TES buffer, pH 7.2. All activities were obtained from the same batch of enzyme preparation. The nl ethylene shown in the table was the absolute amount of ethylene evolved.

a) The presence of other enzymes capable of converting those substrates to etylene via acrylate,

b) a broad specificity of the enzyme that converts acrylate to ethylene.

Ethylene synthesis from β-alanine would be expected to be lower than that from acrylate, since the latter compound is the immediate precursor for ethylene production by this pathway. The results obtained from gel electrophoresis in an alkaline system showed that there were at least four bands of protein present in the preparations. Thus, it is possible that each protein band corresponds to one enzyme of the β-alanine pathway. The enzymes of the β-alanine pathway would seem to emerge from the Sephadex G-100 column as a whole unit of enzyme system.

For conversions of pyruvate and propionate to acrylate, however, the following reaction steps (that have already been studied in microorganisms, higher plants and animals (81, 82, 83)) might be involved:

Pryuvate: $CH_3-\overset{O}{\overset{\|}{C}}-COOH \xrightarrow{+\ 2\ H^+} CH_3-CH(OH)-COOH \rightleftharpoons (CH_2OH-CH_2-COOH)$

$(CH_2OH-CH_2-COOH) \longrightarrow CH_2=CH-COOH$

Propionate: (1) $CH_3-CH_2-COOH \xrightarrow{-\ 2H^+} CH_2=CH-COOH$

or (2) $CH_3-CH_2-COOH \xrightarrow{[O]} CH_2OH-CH_2-COOH \longrightarrow CH_2=CH-COOH$

For all these conversions that have been found in higher plants, microorganisms and animals, however, the starting compounds were first converted to CoA derivatives and the product of such conversions was acrylyl CoA. (The oxidation of propionate by reaction (2) above is an exception.) In the present study, ATP and CoA were present in the assay mixture. It is possible therefore that the pyruvate and propionate were converted first to the CoA derivatives in the reaction mixtures.

The fact that gel electrophoresis in an alkaline and in an acidic medium revealed not more than 4 protein bands points to a possible broad specificity of some enzymes of the β-alanine pathway. The dehydrogenase that catalyzes the conversion of malonic semialdehyde to β-hydroxypropionate,

$$CHO\text{-}CH_2\text{-}COOH \underset{-\,2H^+}{\overset{+\,2H^+}{\rightleftharpoons}} CH_2OH\text{-}CH_2\text{-}COOH$$

could perhaps catalyze addition of $2[H^+]$ to pyruvate to form α-hydroxypropionate, and a removal of $2[H^+]$ from propionate to yield acrylate. The enzyme for dehydration of β-hydroxypropionate to acrylate would perhaps catalyze a dehydration of α-hydroxypropionate to yield acrylate. The difference in activity of the enzyme preparations towards the above substrates (i.e. in terms of specificity of the enzymes), would result in differences in ethylene production from the various substrates.

It may be pointed out here that the detection of only four bands of proteins by gel electrophoresis does not necessarily mean that there were only four protein components of the purified enzyme preparations. Since one of the properties of proteins is charges on their surfaces, there would always be a possibility of interaction among different types of the protein molecules of the enzyme preparations. Where these occurred, more than one type of protein could migrate together in an applied electric field. Such interactions, however, would be dependent on the pH and ionic strength of the buffer medium. In the experiments that were done, two buffers of different pH and ionic strength were used, therefore, the probability of resolving such components further would have been increased.

An alternative explanation that exists for the ethylene production from pyruvate and propionate by the enzyme preparations, catalyzing ethylene production from acrylate, would be a broad specificity of the preparations. It is very unlikely, however, that the product of decarboxylation of those two compounds would be ethylene. (Meheriuk and Spencer (84) and Thompson (56) have already provided evidence for involvement of a decarboxylation step in the ethylene production from β-alanine by enzyme preparations of tomatoes and wax bean cotyledons. They proposed this decarboxylation reaction for the last reaction step of the β-alanine pathway leading to formation of ethylene from acrylate). When acrylate was replaced with pyruvate, the product of decarboxylation would be acetaldehyde:

$$CH_3\text{-}\overset{\overset{O}{\|}}{C}\text{-}COOH \longrightarrow CH_3\text{-}CHO + CO_2$$

and replacement of acrylate with propionate would give ethane:

$$CH_3\text{-}CH_2\text{-}COOH \longrightarrow CH_3\text{-}CH_3 + CO_2$$

If acetaldehyde were produced from pyruvate, it would have to be converted further to ethylene. Shimokawa and Kasai (32) proposed an acetaldehyde-cysteine complex, presumably S-ethylcysteine, as a direct precursor of ethylene in apple particles (sedimented at 13,000 g for 20 minutes). Further work led them to propose the formation of ethylene from the ethyl moiety of S-ethylcysteine in the presence of flavin mononucleotide (FMN) and light. In the presence of FMN in darkness, they obtained no ethylene. In the present study, a sulfhydryl compound, DTT and coenzyme A, were present but the reaction flasks were shielded from light by aluminium foil. Therefore, the ethylene production from pyruvate seemed to occur through another mechanism. In any case, at least one enzymatic step would be required for the conversion of pyruvate to ethylene since the assay mixture in the absence of the enzyme preparation evolved no ethylene.

In the case of propionate, no ethane was detected, although it is possible that the intermediate of the decarboxylation of propionate could remain bound to the enzyme and undergo conversion to the detectable amount of ethylene.

With regards to the ethylene production from L-methionine, it is not surprising to find that only trace amount of ethylene was produced from the amino-acid by the enzyme preparation. Although methionine at 5 mM was used by Mapson (29) and Lieberman et al (31),

their assay conditions as well as the enzyme systems were quite different. The finding of Stinson (42) that ethylene was evolved from the soluble enzyme preparations of wax bean cotyledons, in the presence of all the cofactors (all at the same concentrations as used in the present study) but 5 mM in L-methionine, does not contradict the present finding (although Stinson found such ethylene formation to be partially nonenzymatic), since the preparations used by Stinson were less purified. There is no information from his work regarding use of L-methionine at a lower concentration than 5 mM. If 5 mM in L-methionine were to be used for the purified enzyme preparation in the present study to obtain an appreciable amount of ethylene, it could still be concluded that the enzyme preparations in this present study do not utilize L-methionine as effectively as do the other 'potential precursors' (acrylate, pyruvate and propionate).

4) Requirement for coenzyme A

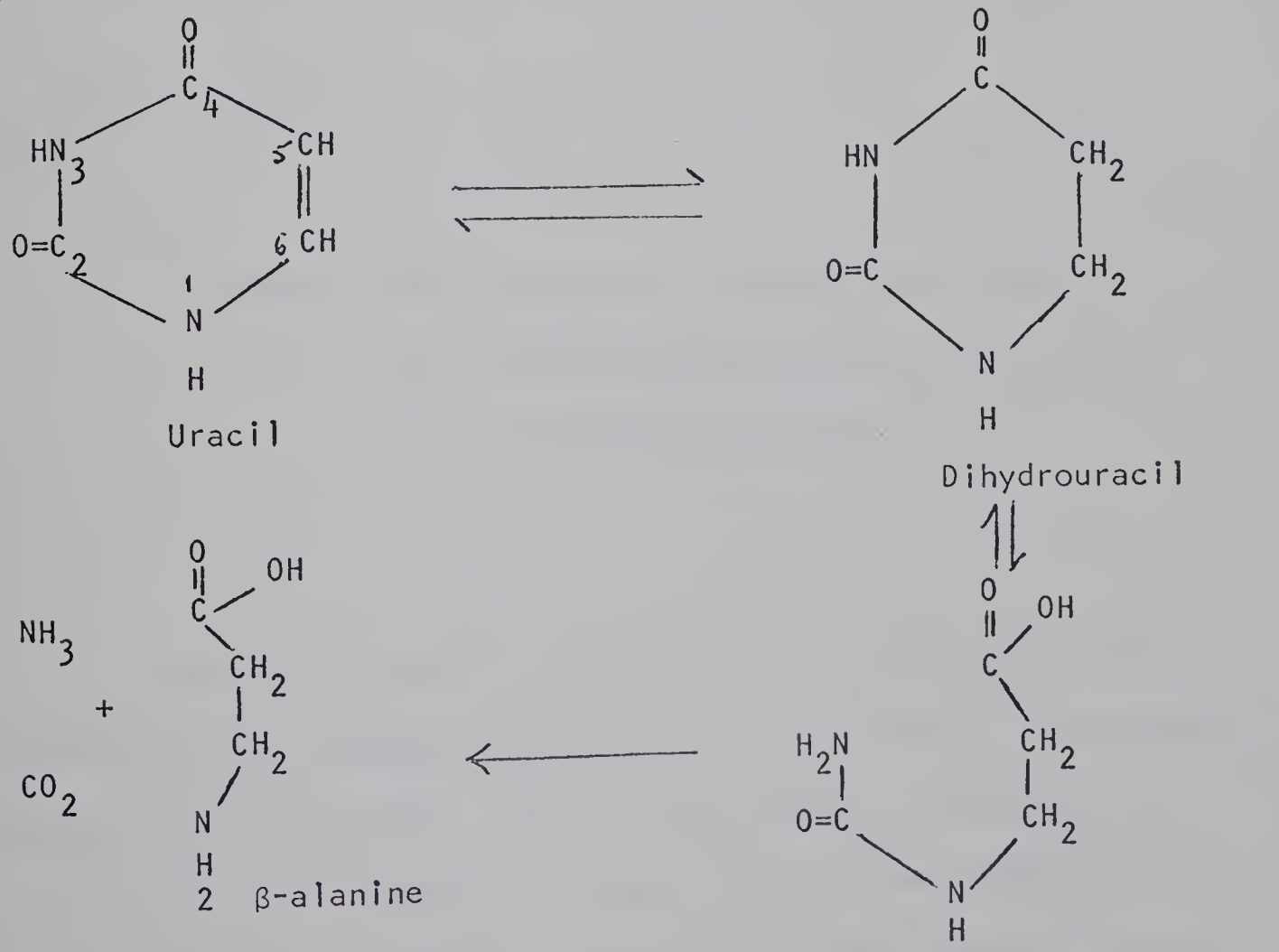
Omission of coenzyme A from the complete assay mixture (in the presence of acrylate) caused a marked decrease in ethylene production by the purified enzyme preparation (nl ethylene/mg. protein dropped from 1.80 to 0.2 in its absence). Stimulation of ethylene production by addition of coenzyme A, ATP and Mg^{++} to enzyme powders of wax bean cotyledons was observed by Thompson (56). In his study, the concentration of coenzyme A was 0.17 mM. Although no direct evidence, such as an identification of the coenzyme A derivative of acrylate, was provided in the present study, there still exists

a possibility of conversion of acrylate to acrylyl CoA, a reaction that would be catalyzed by a thiokinase. (Evidence for requirement of ATP in ethylene biosynthesis of enzyme preparations from wax bean cotyledons has been provided by Thompson (56) and Stinson (42)).

Section E: Methionine and Ethylene Biosynthesis in detached Bean Leaves

Previous work done in this laboratory has shown that detached leaves from the bean plants at the same age and grown under similar conditions could metabolize both L-methionine and β-alanine (see p. 14). Knight (2) showed that β-alanine was formed from uracil via reaction steps shown in fig. 9 (A). It was of interest to see if a relationship could be established for the production of ethylene from L-methionine and β-alanine, possibly through a portion of the pathway in fig. 9 (B).

(A)



(B)

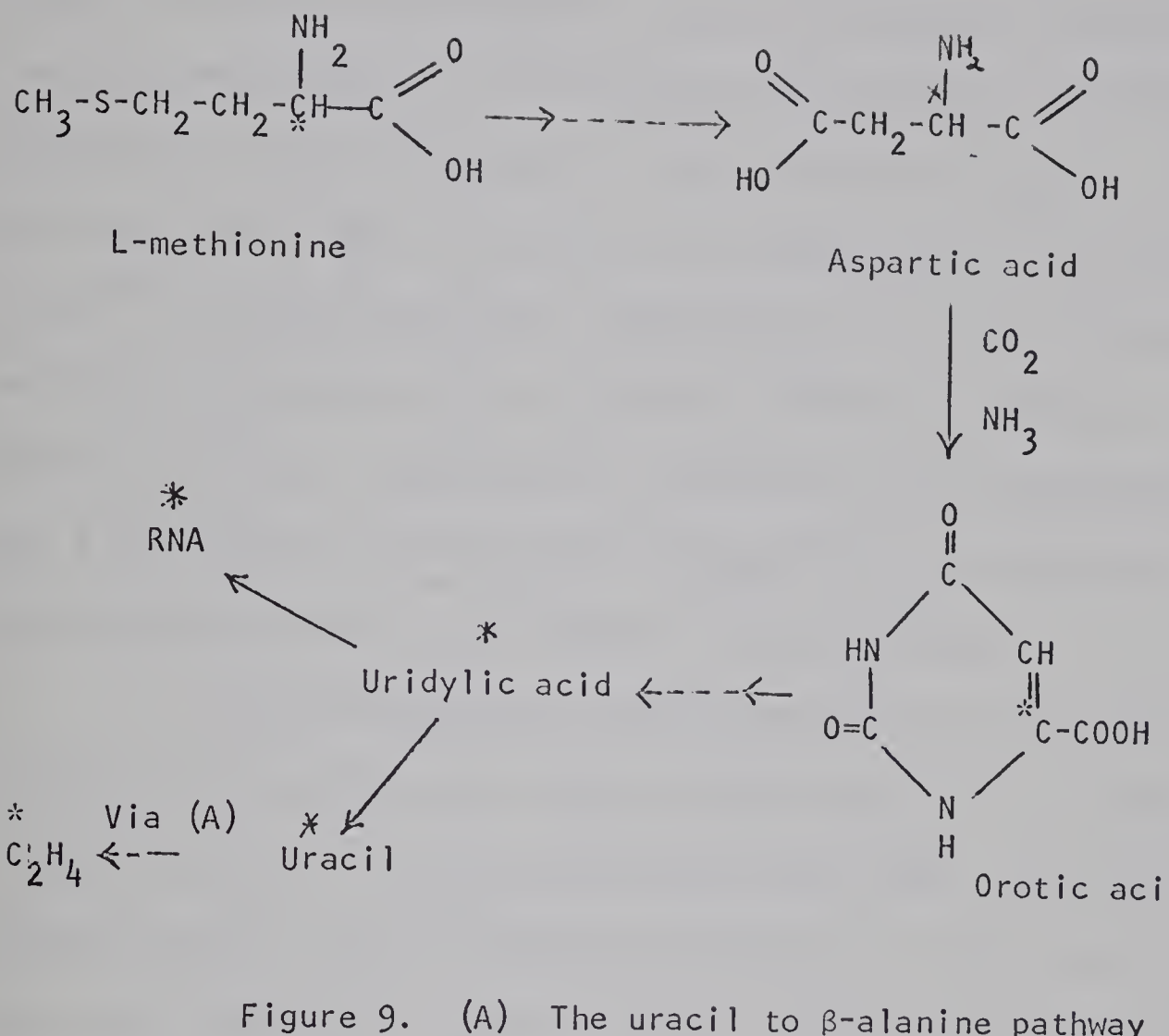


Figure 9. (A) The uracil to β-alanine pathway

(b) Possible pathway for conversion of L-methionine to uracil

Uniformly labeled L-methionine, if it were metabolized according to this pathway, should give rise to labeled intermediates indicated in figure 9 (B). Any of these labeled intermediates that accumulated in sufficient amounts should be detectable. On the other hand, methyl-labeled L-methionine would not give rise to labeled ethylene by this pathway. Lieberman et al (15) from post-

climacteric apple tissue slices and Burg and Claggett (16) from apple tissue slices of unstated stage of development claimed that insignificant amount of [^{14}C]-ethylene was produced from [$^{14}CH_3$]-L-methionine. The former group of workers obtained 176 dpm in [^{14}C]-ethylene from [$^{14}CH_3$]-L-methionine and 34,556 dpm in [^{14}C]-ethylene from [^{14}C-3,4]-DL-methionine. Radioactivity of the two labeled methionine samples was 2.5 μc_i (5.55×10^6 dpm) and 13.5 μc_i (29.97×10^6 dpm) respectively. The latter workers obtained 29 dpm [^{14}C]-ethylene from 147.85×10^6 dpm [$^{14}CH_3$]-L-methionine, and 36,500 dpm from 54.17×10^6 dpm [U-^{14}C]-L-methionine.

The following tracer studies were done on four leaf samples from plants grown under conditions on p. 17. Knight (2) found that leaves from the bean plants grown under such conditions showed the first peak of ethylene evolution at a much earlier date than those obtained from plants grown under the conditions chosen by Stinson (42). [The peak appears at day 7 in the former case, and at day 19 in the latter case]. The number given to each leaf sample will be used for that particular sample throughout this section.

1) Ethylene production from detached bean leaves fed with radioactive L-methionine.

Both [U-^{14}C]-L-methionine and [$^{14}CH_3$]-L-methionine could be utilized to form ethylene but the percentage of conversion was greater from the former compound (table 13).

Table 13

Conversion of radioactive methionine to ethylene by bean leaves

Sample No.	Radioactive methionine and dpm administered	Collection period (hr)	Ethylene dpm	Ethylene nl	Percentage of Conversion	Sp. activity C_2H_4/ Sp. Activity methionine
I	0.146 mM [U-^{14}C]-L-methionine 44.4 x 10^6 dpm (Sp. ac. 222 mc$_i$/mmole)	0-4	1.42x10^6	117.0	3.2%	0.63
II	1.57 mM [$^{14}CH_3$]-L-Methionine 22.2 x 10^6 dpm (Sp. ac. 10.66 mc$_i$/mmole)	0-4	8.41x10^3	70.5	0.4%	0.13
III	0.073 mM [U-^{14}C]-L-Methionine	0-2	3.36x10^4	45		
	22.2 x 10^6 cpm (sp. ac. 222 mc$_i$/mmole)	2-4	3.28x10^5	45	1.65%	0.25
IV	0.05 mM [U-^{14}C]-L-Methionine * 15.54 x 10^6 dpm (Sp. ac. 222 mc$_i$/mmole)	0-2	4.76x10^4	39	0.5%	0.48

0.6 ml. of radioactive methionine solution was administered to detached bean leaves (see materials and methods p. 18). The ethylene collections were started as soon as leaf petioles were placed in the methionine solutions.

* After the collection period of 0-2 hr., radioactivity of the remaining methionine solution that had not been taken up by leaves was determined in a scintillation counter (Nuclear-Chicago, Unilux II.) and found to be 5.92 x 10^6 dpm.

In these experiments, however, one should take into consideration the difference in radioactive specific activity of the two forms of labeled methionine, since this gives a difference in concentrations of the two radioactive solutions that have the same total radioactivity.

For administration of 22.2×10^6 dpm, use of [U-^{14}C]-L-methionine whose specific activity was 222 mc_i/mmole to prepare the radioactive solution required addition of only 0.05 µmole L-methonine. If the [$^{14}CH_3$]-L-methionine (specific activity 10.66 mc_i/mmole) was used, 1 µmole of the compound would be required for 22.2×10^6 dpm. At a higher concentration of the amino acid, the probability of its being utilized by enzymes for other reactions would be greater. (This is true only for those enzymes that have a greater K_m value than the enzyme utilizing L-methionine for ethylene production). Therefore, if the 'CH_3' group of L-methionine was directly converted to ethylene, such a possibility would be an explanation for a low conversion of the compound to ethylene, relative to the conversion obtained with [U-^{14}C]-L-methionine.

A possible alternative explanation would be that the ethylene was formed directly from carbon atoms, other than the carbon atom of the terminal methyl group, of the L-methionine molecule. An indirect conversion of the terminal methyl group to ethylene, and perhaps via many slow reaction steps, would result in a much slower rate and extent of the conversion.

A third possible explanation is that since the leaf sample number II that was fed with [$^{14}CH_3$]-L-methionine had a lower ethylene synthesizing activity than the leaf sample numbers I and III (Leaf sample number II evolved 70.5 nl ethylene over 0 - 4 hours, whereas leaf sample number I and III evolved 117.0 nl and 90.0 nl ethylene respectively), the ability to utilize the L-methionine administered would be smaller. Leaf sample number IV that also had a relatively low ethylene synthesizing activity (evolved 39 nl ethylene over 0 - 2 hours), however, converted as much as 0.5% of [U-^{14}C]-L-methionine within the collection period of 2 hours (table 13). Thus, the low conversion of [$^{14}CH_3$]-L-methionine to [^{14}C]-ethylene by leaf sample number II would not likely be the result of a difference in ethylene synthesizing activity of the leaf samples.

The difference in percentage of conversion between leaf sample number I and number III, on the other hand, could be a result of a difference in the ethylene synthesizing activity of the samples. From these two samples, ethylene that was produced was 117.0 nl and 90.0 nl respectively over 0 - 4 hours, thus, indicating a higher activity of the leaf sample number I in ethylene production.

2) Carbon dioxide production from radioactive methionine administered to bean leaves.

As expected, leaf sample number II that was fed with [$^{14}CH_3$]-L-methionine had the greatest recovery of ^{14}C in carbon dioxide (table 14). Between the two leaf samples fed with [U-^{14}C]-L-methionine for 4 hours (i.e. leaf sample numbers I and III) leaf

Table 14

Carbon dioxide production from

the feeding experiments with methionine

Sample No.	Radioactive methionine and dpm administered	Collection period (hr)	dpm CO_2	Percentage of recovery of ^{14}C in CO_2
I	0.146 mM [U-^{14}C]-L-methionine 44.4×10^6 dpm (sp. ac. 222 mc_i/mmole)	0-4	6.93×10^5	1.56
II	1.57 mM [$^{14}CH_3$]-L-methionine 22.2×10^6 dpm (Sp. ac. 10.66 mc_i/mmole)	0-4	21.02×10^5	9.47
III	0.073 mM [U-^{14}C]-L-methionine 22.2×10^6 dpm (sp. ac. 222 mc_i/mmole)	0-2 2-4	4.61×10^5	2.08
IV	0.050 mM [U-^{14}C]-L-methionine 15.54×10^6 dpm (sp. ac. 222 mc_i/mmole)	0-2	4.60×10^5	4.78

0.6 ml. of radioactive methionine solution was administered to detached bean leaves. The ethylene and the CO_2 collections were started as soon as leaf petioles were placed in the methionine solutions.

sample number I that was more active in producing ethylene, produced less $^{14}CO_2$ than leaf sample number III. One possible explanation for such difference is that the ethylene synthesizing system from methionine was competing with other systems that could utilize the amino acid. Since leaf sample number I was more actively producing ethylene, [U-^{14}C]-L-methionine would be metabolized more efficiently in the former system if this system were a major one involved in ethylene production. Another possible explanation would be that the rate of turnover of $^{14}CO_2$ was higher in leaf sample number I than that in number III, thus, an apparent lower percentage of recovery of ^{14}C in $^{14}CO_2$.

Leaf sample number IV that was fed for 2 hours with [U-^{14}C]-L-methionine produced most $^{14}CO_2$ among the three samples fed with [U-^{14}C]-L-methionine (percentage of conversion to [^{14}C]-ethylene was the lowest, however). It may be concluded therefore that the metabolism of [U-^{14}C]-L-methionine into $^{14}CO_2$ was more important during the early period of time after the leaves had been placed in the solution of radioactive amino acid. In a later stage, however, the conversion of the amino acid to [^{14}C]-ethylene became more important (sample III, table 13).

3) Distribution of ^{14}C among the compounds of leaf extracts

a) Identification of the compound by TLC

Two peaks of radioactivity were detected by the actigraph. Since the actigraph was so set that compounds with low radioactivity (below 3,000 counts per min. for leaf sample number I and III;

and below 10,000 counts per min. for leaf sample number 11) would not be detected, the radioactive compounds that were identified would correspond to the products of metabolism of radioactive methionine that had accumulated most radioactivity (see p. 93 for the identities of these compounds).

With regards to the separation ability of the solvent system, Gordon, Thornburg and Werum (62) found the system to be satisfactory for use as the first stage purification of unknown biologically active or radioactive compounds. They found, however, that the R_f values could be affected by the quantity of substance in the initial spot and by the kind and amount of other substances present. A difference of 5 or more units, higher or lower than that of pure compounds, in R_f values could be expected (62). Interaction between radioactive compounds was found by the present author. This finding could be an explanation for difference in resolution pattern from that of Knight (2).

When a mixture of 2 µl [^{14}C-2]-β-alanine (sp. ac. 4 mc_i/mmole); 3 µl of [U-^{14}C]-L-methionine (sp. ac. 222 mc_i/mmole); 5 µl of [^{14}C-2]-uracil (sp. ac 6.25 mc_i/mmole) and 5 µl of [^{14}C-2]-orotic acid (sp. ac. 24.3 mc_i/mmole) was spotted as a band on a cellulose plate, followed by separate spots of 10 µl of β-alanine (1 mg/ml); methionine (1 mg/ml); uracil (1.21 mg/ml) and orotic acid (1.2 mg/ml), and the plate was developed with the solvent system that was

used for the leaf extracts, only two peaks of radioactivity were detected by the actigraph. These gave the R_f values of 0.29 and 0.61. When the plate was examined under a UV lamp, four spots of the standard compounds were observed, which had the following R_f values: 0.24, 0.56; 0.64 and 0.74 corresponding to β-alanine, methionine, orotic acid and uracil respectively. Further separation and identification of the radioactive components of each peak of radioactivity (by use of plates and solvent systems as in table 16) showed that β-alanine and methionine were chromatographed together and located in peak no. I of the cellulose plate developed with the solvent system in table 16.

With this finding, and further separation of each peak of radioactivity in table 16, the three major components of the leaf extracts were identified as methionine, orotic acid and uracil. In terms of the peak heights, methionine and uracil had about the same peak height. This was about three times as high as that of orotic acid. The detection of radioactive methionine in all the three leaf extractsindicated that a large amount of the radioactive amino acid that had been taken up by leaves, was metabolized at a slow rate. An alternative explanation would be that the turnover rate of the amino acid was high. As soon as it was metabolized to other compounds, part of the molecule that could be converted back to methionine, e.g. the methyl end group could be used for synthesis of more methionine.

For the synthesis of orotic acid, CO_2, NH_3 and ATP are

Table 15

R_f values of peaks of radioactive compounds separated by TLC on cellulose

Sample No.	R_f values 1st peak	2nd peak
I	0.23	0.56
II	0.30	0.65
III	0.26	0.61

100 µl leaf extract was spotted as a band on a TLC plate coated with cellulose and developed with solvent system containing isopropanol-pyridine-water-acetic acid (8:8:4:1 v/v/v/v). Peaks of radioactive compounds were detected with an actigraph III (for the settings on the actigraph see p. 30). The lower limit of counts that would be detectable by the actigraph was 3,000 for leaf sample number I and III, but was 10,000 for leaf sample number II.

Table 16

R_f values of radioactive compounds determined by rechomatography on a thin-layer plate

Sample No.	*Peak No.	TLC plates	R_f values in $EtOH/NH_3$	R_f values in $nBuOH/MeOH/H_2O/NH_3$
I	1	Cellulose	Not done	0.17
		Silica gel G	0.68	Not done
	2	Cellulose	Not done	0.16 and 0.42
		Silica gel G	0.68	Not done
II	1	Cellulose	Not done	0.27
		Silica gel G	0.80	Not done
	2	Cellulose	Not done	0.29 and 0.47
		Silica gel G	0.85	Not done
III	1	Cellulose	Not done	0.14
		Silica gel G	0.73	Not done
	2	Cellulose	Not done	0.14 and 0.45
		Silica gel G	0.86	Not done

Variation in R_f values of standard samples (see p. 93) was $\pm$ 10%. *Peaks of radioactivity corresponded to peak no. I and II as determined in table 15. The compounds were identified by chromatography with standard radioactive compounds.

required as precursors, as well as aspartic acid for completion of the ring structure. The results obtained in this study seem to suggest that

i) carbon dioxide that was produced from both [$^{14}CH_3$]-L-methionine and [U-^{14}C]-L-methionine could be utilized for the synthesis of orotic acid

ii) in the case of [U-^{14}C]- -methionine, the amino acid could possibly be converted to aspartic acid which was then utilized for the synthesis of orotic acid.

In both cases, subsequent conversion of orotic acid to uracil would give rise to radioactive uracil. When uracil was broken down to β-alanine and ethylene (as found by Knight (2)), however, only the part of the uracil molecule that was synthesized from labeled aspartate would give rise to radioactive ethylene. (For the structure of these compounds and steps for their conversion to ethylene see fig. 9).

In the present experiments, no radioactive β-alanine was detected. The amino acid could be present in too low a quantity to be detected by the actigraph. (In the feeding experiment of [^{14}C-6]-orotic acid to bean leaves, Knight (2) found a low percentage of conversion into [^{14}C] β-alanine in older leaves where a peak of ethylene production was obtained). In contrast to this is the finding of high radioactivity in uracil (also found by Knight (2) in his feeding experiment with [^{14}C-6]-orotic acid), indicating a high uracil synthesizing activity of bean leaves at this stage of

development. Since Knight (2) found that β-alanine was synthesized from uracil, the dilution effect from large endogenous uracil pool, could result in no detectable [^{14}C]-β-alanine. Alternatively, a slow conversion of uracil to β-alanine but a rapid conversion of β-alanine to ethylene could be an explanation for the nondetectable [^{14}C]-β-alanine.

b) The recovery of ^{14}C in RNA fraction

Among the three leaf samples, leaves from a leaf sample that was fed with [$^{14}CH_3$]-L-methionine showed the greatest recovery of ^{14}C in the RNA extract. Although there was a possibility of this ^{14}C being incorporated into the sugar portion of the nucleotides, the separation of labeled compounds on cellulose plates showed peaks of radioactive orotic acid and uracil. Therefore, one might assume the presence of ^{14}C in the base portion of the RNA nucleotides.

Table 17

Radioactivity recovered in the RNA faction

Sample No.	Methionine and dpm fed	dpm in the RNA fraction	Percentage of ^{14}C recovery of ^{14}C in the RNA fraction
I	0.146 mM [U-^{14}C]-L-methionine 44.4×10^6 dpm (Sp. ac. 222 mc_i/mmole)	1.67×10^6	3.8%
II	1.57 mM [$^{14}CH_3$]-L-methionine 22.2×10^6 dpm (Sp. ac. 10.66 mc_i/mmole)	6.35×10^6	29%
III	0.073 mM [U-^{14}C]-L-methionine 22.2×10^6 dpm (Sp. ac. 222 mc_i/mmole)	1.82×10^6	8.2%

0.6 ml. of radioactive methionine solution was administered to detached bean leaves. After feeding period of 0-4 hr., ribonucleic acid fraction was extracted (see material and methods p. 29), and radioactivity of the fraction was determined by counting in a Nuclear Chicago Unilux II, Liquid Scintillation counter.

CONCLUSIONS

An enzyme preparation, made soluble by treatment of the particulate 32,000 g fraction of waxbean cotyledons with Triton X-100, was found to catalyze ethylene production from acrylate. The presence of a sulfhydryl protecting compound, dithiothreitol (DTT), was shown to be essential for this activity. The concentration of DTT to be added to the assay medium was so selected that its presence did not affect the enzyme preparations in other ways. (It appeared that a high DTT:protein ratio resulted in an inhibition of ethylene production by the enzyme preparation. Preparations that produced a considerable amount of ethylene from endogenous compounds showed an increase in ethane production with an increase in concentration of DTT). It was found that 6.5 mM DTT was a suitable concentration to add to an assay mixture containing about 60 mg protein.

Within a range of concentrations between 0.5 mM and 2.0 mM in the reaction mixture, 1.0 mM acrylate was found to give a maximal stimulation of ethylene production by the soluble enzyme preparations. The ethylene produced in the presence of 1.0 mM acrylate increased linearly with increase in enzyme concentration between 4.6-7 mg protein/ml assay mixture. At the enzyme concentration of 10 mg/ml, the ethylene production was decreased appreciably. This may be indicative of the presence of other systems that could compete with the ethylene synthesizing system for acrylate.

Alternatively, the decrease in ethylene production in the presence of large amount of protein sample could be an indication of a presence in the protein sample of some inhibitors of the system synthesizing ethylene.

The choice of further purification of the soluble enzyme preparations by gel chromatography on Sephadex G-100 is justified by the following points:

1) Although the preparations that were passed through the Sephadex G-100 column did not emerge from the column as distinct, separable peaks (which absorbed light at 254 nm), the ethylene synthesizing activity of the soluble enzyme preparation could be localized in a small portion of the whole protein peak. In contrast to this, when ammonium sulphate was used, appreciable ethylene synthesizing activity was obtained in all the fractions that were salted out; a relatively high ethylene synthesizing activity was localized in the fraction that was precipitated by saturation with 40-70% ammonium sulphate.

2) The endogenous ethylene producing activity that was always obtained from the soluble enzyme preparations was not detected from the purified enzyme preparations from Sephadex G-100 column. It seems therefore that most of the endogenous compounds or systems that could be involved in ethylene synthesis had been removed by this treatment. The endogenous activity of preparations obtained by purification with ammonium sulphate, on the other hand, was greater than that of the original soluble enzyme preparations.

Demorest and Stahmann(40) reported that protein that could not be utilized for ethylene production, after treatment with a proteolytic enzyme pronase, could stimulate ethylene production in the presence of peroxidase. They suggested the role of pronase was to release the C-terminal methionine, which could then be utilized for ethylene production. Their report is very interesting especially in view of the present finding that ammonium sulphate treated enzyme preparation had a higher endogenous ethylene synthesizing activity than that of the original sample. The presence of NH_4^+ in the preparation could activate the proteolytic enzyme (Tadashi and Murachi (85) found that trypsin activity (commercially supplied) could be activated by $(NH_4)^+$), thus causing an increase in endogenous activity of the preparations. If the endogenous activity was caused by L-methionine, there would be two enzyme systems present in the preparations, one that utilized L-methionine and was removed by gel chromatography on the Sephadex G-100.

3) The purified enzyme preparations from Sephadex G-100 column produced little ethane from the assay mixture, either in the presence or absence of acrylate. The soluble enzyme preparations that were subjected to heating at 50° and above, in the present study, produced a considerable amount of ethane. It was probable that activity of the preparations had been modified by heating, since ethane is not normally produced by whole bean cotyledons. Consequently, a choice for purification of the preparations by heating to denature the heat sensitive proteins would perhaps not be

appropriate. (Very little information has been available for ethane production by an *in vitro* system).

4) Purification that was achieved by gel chromatography on Sephadex G-100 was sixfold. ("Purification" is used in terms of amounts of ethylene produced /mg protein in the presence of the complete reaction mixture). The purification achieved by treatment with ammonium sulphate at 40-70% concentration was 2.3 fold.

An interesting observation was that there was an increase in ethylene synthesizing activity from acrylate when the temperature was raised to 50°. Although the author is well aware of differences in many properties of an *in vitro* from an *in vivo* system, the effect of temperature in increasing the production of ethylene might have a significance *in vivo*. Does it mean that the ethylene synthesizing system does not normally operate at its optimal activity? Even if the environmental temperature were 50°, some other enzymes that are involved in providing substrates for ethylene production, and are more sensitive to high temperature, would become inactivated in time, with a resulting apparent loss in ability to synthesize ethylene by the tissues. (Burg (6) and other workers (86, 87) reported that many fruits, if not all, are unable to produce ethylene at temperatures above approximately 35°). No information is available, at present, for effect of temperatures on ethylene production by other tissues, except that of Meheriuk (75) on subcellular systems from tomatoes.

The second interesting point is the broad specificity of the purified enzyme preparations for substrates. (It was found that the enzyme preparations could utilize acrylate, pyruvate, propionate and β-alanine for ethylene production.) An emphasis should be made here, however, on the purity of these preparations, since they are not highly purified. In addition, it is of significance that bean leaves could metabolize L-methionine to orotic acid, uracil and ethylene. If such conversion of L-methionine to uracil also occurs in bean cotyledons, the scheme such as that in figure 10 may now be drawn to relate interconversion steps between precursors studied in this present work and those, which have been proposed by other workers, for ethylene biosynthesis. The extent of occurrence of any part of the whole scheme would depend on stages of development and types of the tissues.

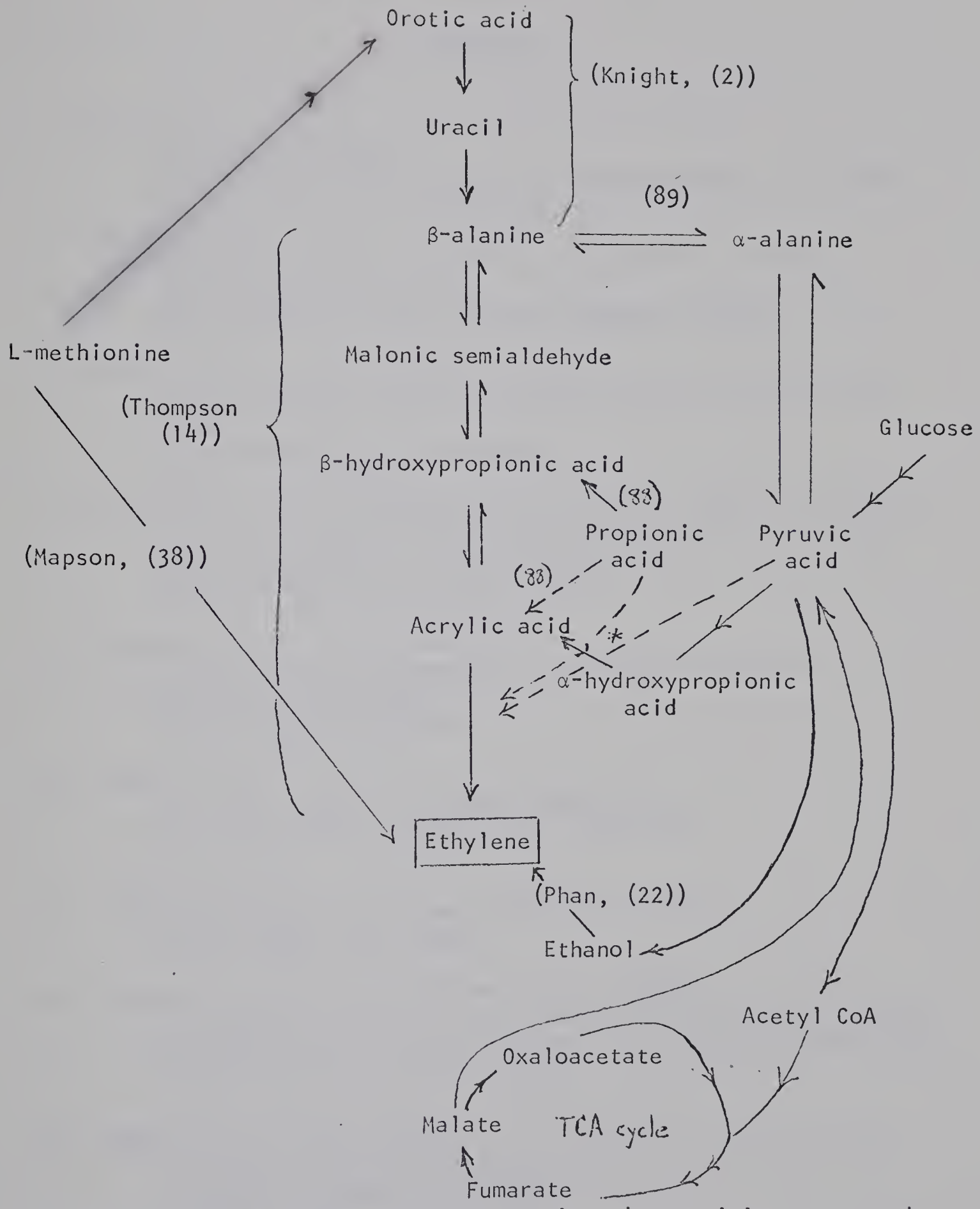


Figure 10. Interrelationship between various 'potential precursors' for ethylene biosynthesis.

→→ represents pathway involving more than one reaction step

- - -→ represents an alternative route for conversion

* represents reaction speculated in the present study

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